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TOXICITY OF NATURAL COMPOUNDS ON PLANTS
AND FUNGI

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Introduzione

Le variazioni delle proprietà del suolo causate dalle piante e che a loro volta influenzano la crescita vegetale sono dette in lingua anglosassone plant–soil feedbacks (Bever et al., 1997; Wardle, 2002; Ehrenfeld et al. 2005; Kulmatiski & Kardol 2008).

Tra i plant-soil feedback riportati in letteratura alcuni sono negativi e detti appunto negative plant-soil feedback (NF) (Klironomos, 2002; Bever 2003; Kulmatiski et al., 2008; Petermann et al., 2008) in quanto possono provocare la degenerazione della pianta (van der Putten et al., 1988), o la morte delle plantule vicine alla pianta madre (Packer & Clay, 2000; Mangan et al., 2010).

Il NF è un importante fattore per l'equilibrio delle comunità vegetali naturali (van der Putten et al., 1993) e per la coesistenza tra specie (Bever et al., 1997, Bonanomi et al., 2005a, 2005b): è stato osservato nelle praterie (Reynolds et al., 2003), nelle foreste temperate e tropicali (Mangan et al., 2010) e negli agro-ecosistemi (Singh et al., 1999).

Comprendere i meccanismi del NF negli agroecosistemi è un passo importante per individuare le cause della stanchezza del suolo.

Il NF è stato considerato dipendente dall'esaurimento dei nutrienti del suolo (Ehrenfeld et al., 2005) e dalla densità e composizione delle popolazioni di agenti patogeni (Packer & Clay, 2000; Klironomos, 2002; Kardol et al., 2007). Tuttavia, lo studio della stanchezza del terreno in agricoltura (Zucconi, 2003) e dei NF in ecosistemi naturali (Bonanomi et al., 2011a) mostra esempi di inibizione specie-specifica non correlati con la disponibilità di nutrienti né con la presenza di patogeni terricoli.

Un'altra ipotesi propone come possibile causa del NF le allelopatie e il rilascio di sostanze fitotossiche dagli essudati radicali e dalla decomposizione della lettiera. Tra queste tossine ci sono acidi organici solubili in acqua, aldeidi alifati, lattoni, acidi grassi a lunga catena, naftochinoni, antrachinoni, fenoli, acidi benzoici e cinnamici, cumarine, tannini, terpeni, steroidi, alcaloidi, acido cianidrico, solfuri, glicosidi e purine. Esiste una letteratura molto ampia sull'individuazione di sostanze fitotossiche di questo tipo (Singh et al., 1999; Yu et al., 2000; van de Voorde et al., 2012; Grodzinsky, 2006; Kulmatiski et al., 2008; Bonanomi et al., 2011a; Miller et al., 1988; Dornbos et al., 1990; Chung et al., 2000; Chon et al., 2002; Börner, 1959; Hartung et al., 1990; Miller et al., 1991; Asaduzzaman et al., 2012; Asaduzzaman & Asao, 2012; Kong et al., 2008; Burger & Small, 1983; Chou & Waller, 1980; Yu & Matsui, 1994; Chen et al., 2011b; Zhang et al., 2010a; b; c; Asao et al., 2004a; b; Gog et al., 2005; Yu and Matsui, 1999; Patrick, 1955;

Patrick and Koch, 1958; Li et al., 2012; Chou & Lin, 1976; Ruan et al., 2011; Kitazawa et al., 2005; Asao et al., 2003; Cao et al., 2011; Yu & Matsui, 1993; Guenzi & McCalla, 1966a, 1966b; Lodhi et al., 1987).

L'autotossicità in piante di pesco è attribuita, per esempio, all'amigdalina, che viene frammentata dai microbi in sostanze tossiche come l'acido cianidrico, causando lesioni alle giovani piante di pesco in reimpianto (Patrick, 1955).

Queste tossine sono velocemente degradate dall'attività microbica del suolo (Blum, 1998; Blum et al., 2000; Weidenhamer et. al., 2013) tanto che gli effetti inibitori della lettiera sono evidenti solo nelle prime fasi di decomposizione (Zucconi, 2003; Bonanomi et al., 2011a). Per questo motivo, l'ipotesi che la causa del NF sia attribuibile alle sostanze rilasciate da lettiera e sostanza organica viene messa in discussione (Fitter 2003; Harper, 2010).

In questo lavoro di tesi viene presentata un'applicazione classica di studi di tossine che prevede l'isolamento, la caratterizzazione e i biosaggi di tossicità di una molecola, prodotta da un organismo, su altri organismi dannosi. In particolare, tale approccio è stato applicato per l'isolamento e caratterizzazione di due lipopeptidi dal ceppo B05A di *Bacillus amyloliquefaciens*, che sono stati testati per la loro attività antifungina su funghi patogeni di piante.

Un secondo lavoro ha invece riguardato un'estensiva analisi della fitotossicità delle lettieri di diverse specie a diversi gradi di decomposizione e la verifica dell'ipotesi che l'autotossicità potesse dipendere da DNA extracellulare.

Infine, un'ulteriore sperimentazione ha verificato la presenza dell'effetto inibitorio specie-specifico del DNA su organismi diversi da piante, tra cui funghi, batteri, protozoi e insetti.

Nelle conclusioni si discute di come i risultati sperimentali conseguiti presentino interesse per futuri studi sui fenomeni di stanchezza del suolo in agricoltura.

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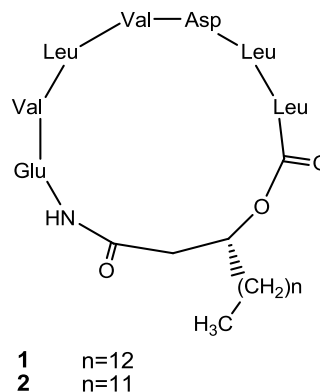
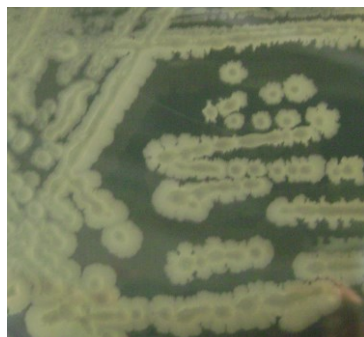
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Chapter 1: Antifungal Tests on Cyclic Lipopeptides from *Bacillus amyloliquefaciens* strain BO5A



Summary

A bioassay-guided fractionation of *Bacillus amyloliquefaciens* strain BO5A afforded the isolation of two new cyclic lipopeptides (**1** and **2**) as the major lipid constituents (> 60 %) of CHCl₃/MeOH (2:1) extract. The chemical structures were elucidated by spectroscopic methods, including NMR spectroscopy, mass spectrometry (MS) and chemical degradation. The compounds are members of the surfactins family and are based on a heptapeptide chain composed by Glu-Val-Leu-Val-Asp-Leu-Leu. Its N-terminal end is N-acylated by a (R)-3-hydroxy fatty acid with linear alkyl chains of 16:0 and 15:0 (**1** and **2**, respectively). The 3-hydroxyl group closes a 25-membered lactone ring with the carboxylic group of the C-terminal amino acid. The isolated compounds were tested for their inhibitory activity against four pathogenic fungi *Fusarium oxysporum*, *Aspergillus niger*, *Botrytis cinerea* and *Penicillium italicum*; and the biocontrol fungus *Trichoderma harzianum*. Compound **2** displayed activity against all tested pathogens.

Introduction

Cyclic lipopeptides are a class of bacterial secondary metabolites based on a polypeptide chain, generally composed of seven amino acids and a 3-hydroxy or 3-amino fatty acid,¹ connected in two places giving rise to a macrocyclic structure. The N-terminal end of the polypeptide is involved in one amide bond with the carboxylic group of the

fatty acid, while the 3-hydroxy or 3-amino group of the fatty acid gives rise to ester or amide bond, respectively, with the C-terminal amino acid.²

Cyclic lipopeptides have received increasing attention as key compounds in biocontrol of plant disease^{3,4} due to their strong antimicrobial activity, low toxicity and low stability, i.e. easily degradable under natural conditions, compared to other chemical pesticides. In this context, many members of the *Bacillus* genus have been studied chemically because they produced lipopeptides which inhibit plant pathogens as well as having a key role in stimulating the host defence mechanism.⁵⁻⁷ Known lipopeptides from *Bacillus* spp. include basic structures - named iturins, surfactins and fengycins - differing in the amino acid composition and sequence, and in the structure of the alkyl chain. In particular, some lipopeptides, isolated from *Bacillus amyloliquefaciens* strain BO7, showed strong and dose-dependent activity against *Fusarium oxysporum*.⁸⁻⁹

It was extended the analysis on strain BO5A of *Bacillus amyloliquefaciens* that afforded the isolation and characterization of two new lipopeptides (**1** and **2**), whose stereostructures have been elucidated by NMR, MS techniques and chemical methods. The isolated compounds were subjected to antifungal tests against the four pathogenic fungi *Fusarium oxysporum*, *Aspergillus niger*, *Botrytis cinerea* and *Penicillium italicum*; and the biocontrol fungus *Trichoderma harzianum*.

Results and Discussion

The cell-free culture filtrate of *B. amyloliquefaciens* strain BO5A was found to contain approximately 0.0015% w/v of CHCl₃-MeOH (2:1) extractable lipopeptides. Purification of these compounds was carried out by reversed-phase HPLC and isocratic elution, affording the new compounds **1** (8.4 mg/L), and **2** (5.8 mg/L). The purity of the isolated compounds, eluted as single peaks, was determined by ¹H NMR spectra (Figure S1).

Compound **1** was isolated as an amorphous solid and showed in the MS spectra peaks at m/z 1037 [M+H]⁺, 1059 [M+Na]⁺ and 1075 [M+K]⁺ (Figure S2) which indicated a molecular formula of C₅₃H₉₃N₇O₁₃. ¹³C NMR data (Figure 1 and Table 1) confirmed the presence of 53 carbon atoms while ¹H NMR data (Figure 1 and Table 1) indicated the lipopeptide nature of the compound with the presence of aminic protons (δ 8.60÷7.50), α-amino acid protons (δ 5.30÷4.00), and a long alkyl chain (δ 0.80÷1.40). In addition, the ¹³C NMR spectrum contained diagnostic signals of carboxyl groups (δ 175.5÷172.1) that,

together with seven nitrogen-bearing carbon signals (δ 50.5–59.8), indicated the presence of seven amide bonds (Figure 1 and Table 1).

Acid hydrolysis of compound **1** (6M HCl, 40h, 110 °C)¹⁰ released the single aminoacids, identified as valine, leucine, glutamic and aspartic acids (2:3:1:1) and the fatty acid 3-methoxy hexadecanoate.

Further information of the chemical structure of **1** was obtained by the fragmentation peaks of tandem MS spectra (Figures 2A/2B and 3A/3B) of compound **1** which were indicative of the consecutive loss of the aminoacid residues in the oligopeptide chain. In particular, fragments at m/z 946 (-113) and 833 (-113) indicated the loss of two residues of Leu from the C-terminal end while further peaks at m/z 718 (-115), 619 (-99) and 506 (-113) indicated the consecutive losses of Asp, Val and Leu, thus showing the peptide chain sequence. This allowed to define the aminoacidic sequence of the compound composed of seven amino acids starting from the N-terminal end as: Glu-Val-Leu-Val-Asp-Leu-Leu. The N- and C-ends are attached to the acidic and alcoholic functional groups, respectively, of a 16 carbons 3-hydroxy acid (3-hydroxy-hexadecanoic acid) (**1**, Figure 1).

Compound **2**, isolated as an amorphous solid, showed a molecular formula of $C_{52}H_{91}N_7O_{13}$ deduced by high-resolution FAB MS measurements, and consistent with the ^{13}C NMR data (Tables 1). MS spectra (Figure S3) showed peaks at m/z 1023 $[M+H]^+$, 1045 $[M+Na]^+$ and 1061 $[M+K]^+$. Preliminary NMR (Figure S4) analysis showed a close similarity between compounds **1** and **2**, indicating the lipopeptide nature of compound **2**. This latter compound had a molecular weight 14 amu lower, thus indicating the absence of a methylene group in compound **2** that could be located either on the alkyl chain or on the peptide chain. Both NMR and MS data indicated that **1** and **2** had the same amino acid sequence and that the difference were related to the fatty acid composed by 15 carbons in lipopeptide **2** instead of 16 carbons in lipopeptide **1**. Thus, the chemical structure of compound **2** is based on the same heptapeptide chain, Glu-Val-Leu-Val-Asp-Leu-Leu, N-acylated to the N-terminal amino acid, Glu, by a 3-hydroxy C_{15} fatty acid with a saturated linear chain (15:0) (Figure 1).^{11,12}

Previous studies revealed a broad antimicrobial activity spectrum of lipopeptides from *Bacillus* species such as *B. subtilis*⁶ and *B. thuringiensis*.¹³ Antifungal strains of *B. amyloliquefaciens* were shown to produce iturins.^{14,15} Only in a one case have surfactins been isolated from strain BO7 of this species, as reported from our previous work⁸ in

which we isolated three surfactin derivatives that showed significant antifungal activity against *Fusarium oxysporum* f.sp. *lycopersici* (*Fol*). Thus, this is the second report of surfactin compounds from *B. amyloliquefaciens*. Following the same methods,⁸ we tested the inhibitory activity of the surfactin **1** and **2** against the same fungus and against some of the most important fungal plant pathogens including the fruit rot agent *Penicillium italicum*, the air-borne pathogens *Aspergillus niger* and *Botrytis cinerea*; and the biocontrol agent *Trichoderma harzianum*.

The data obtained, reported in Figure 4, showed that compound **2** exhibits significant inhibitory activity at three concentrations tested (10, 50 and 100 ppm), with an average mycelial growth reduction of 57%, 43%, 38% and 54% for *Fol*, *P. italicum*, *A. niger* and *T. harzianum*, respectively. Compound **1** was active against *Fol* and *A. niger* with an average inhibition of 59% and 36%, respectively. Neither compound showed activity against *Botrytis*. The mixture of both compounds was less effective than compound **2** alone on all tested fungi (Figure 4).

There have been reports on antifungal activity of bacterial surfactins,⁸⁻⁹ but these compounds are better known for their antiviral and antibacterial activities as well as their surfactant properties.⁴ Although the cellular mechanism of the antifungal mode of action of such compounds is still unknown, their efficacy and availability at low cost make the isolated surfactins attractive potential natural fungicides.

Experimental section

Production of **1** and **2** in *Bacillus* Cell Suspension - Extraction and Purification Procedures

Samples of *Bacillus amyloliquefaciens* strain BO5A were isolated from an orchard soil, located in Larino (Campobasso, Italy) and in particular from the rhizosphere of olive plant, using the methodology described by Boulter et al.¹⁶ and characterized by routine bacteriological tests (Biolog and API test) and by sequencing of the small 16S ribosomal subunit (CBS: Centraalbureau voor Schimmelcultures, The Netherlands). Strain BO5A was routinely cultivated in Minimum Salt Liquid Medium (MSLM: K₂HPO₄ 2.5 g/L; KH₂PO₄ 2.5 g/L; (NH₄)₂HPO₄ 2.5 g/L; MgSO₄ x 7H₂O 0.2 g/L; FeSO₄ x 7H₂O 0.01 g/L; MnSO₄ x 7H₂O 0.007 g/L; sucrose 10 g/L; pH 7.5) at 28 °C and shaken at 120 rpm. Crude culture filtrate was obtained by centrifugation at 14,000 rpm for 5 min and sterilized by filtration (0.22 µm pore size). Bacterial cells were resuspended in sterile PBS

pH 7.4¹⁷ and cell concentration was adjusted spectrophotometrically (610 nm). Bacterial strains were stored at -80 °C with 30% glycerol.

Bacteria cells were removed from the culture by centrifugation at 5000 rpm for 30 min. The lipopeptide fraction was obtained from an aliquot (1L) of cell-free supernatant which has been subjected to acid precipitation, by the addition of 6N HCl to a final pH of 2.0 and incubated overnight at 4 °C. The acid precipitate was recovered by centrifugation at 10.000 rpm for 20 min and lyophilized overnight. The lipopeptides were extracted from the powder by using a CHCl₃-MeOH mixture (2:1, v/v) and passed through a polytetrafluoroethylene (PTFE) filter. The extract was concentrated under vacuum to obtain a crude lipopeptide mixture. The final step in the purification employed high-performance liquid chromatography (HPLC) in isocratic mode on a Varian 940-LC apparatus equipped with refractive index detector. The lipid mixture was chromatographed on an analytical C₁₈ reversed-phase column (Waters μ Bondapak C₁₈ 3.9 x 300mm) eluted with 0.1% of trifluoroacetic acid (TFA) in CH₃CN-H₂O (8:2, v/v) obtaining pure compound **1** (8.4 mg, *t_R*=5.4 min) and **2** (5.8 mg, *t_R*=4.2 min).

Antifungal Assays – Growth Condition and Bioassays with Purified Lipopeptides

Fusarium oxysporum f.sp. *lycopersici* (*Fol*) strain 4287 (FGSC 9935) was isolated from infected tomato plants.¹⁸ *Penicillium italicum* (*PI*), *Aspergillus niger* (*AN*), *Botrytis cinerea* (*BC*), and *Trichoderma harzianum* (*TH*) strain T-22 were obtained from the *Dipartimento di Agraria*, Naples, Italy. These fungal pathogens were cultured on solid potato dextrose agar (PDA, Liofilchem S.p.a., Italy) or in submerged culture at 150 rpm in potato dextrose broth (PDB, prepared from fresh potatoes) for 4 days at 28 °C on a rotary shaker (200 rpm). Conidial suspensions for antifungal assays were prepared as previously reported.¹⁹

The two purified LPs (**1** and **2**) were dissolved in ethanol, alone or in mixture, and added to the medium at a final concentration of 10, 50 and 100 ppm. In mixed applications, each compound was used at equimolecular ratio to produce a final additive concentration of 10, 50 or 100 ppm. The solvent ethanol was used as a negative control. Bioassays were conducted in 96-well plate against conidia of the five chosen fungi (*F. oxysporum* f.sp. *lycopersici*, *P. italicum*, *A. niger*, *T. harzianum* and *B. cinerea*). Samples contained a mixture of purified LPs (or ethanol as a control) mixed with PDB and 10⁵ fungal conidia ml⁻¹. Plates were incubated at 28 °C on a rotary shaker (200 rpm) for different times and fungal growth was determined spectrophotometrically by measuring

absorption at 595 nm, using a Microplate Reader (mod. 550, Bio-Rad). Data from bioassays were submitted to variance analysis (ANOVA) using the SPSS software (version 16.0 for Windows, SPSS Inc. Chicago, Illinois) and means were compared by the Tukey's test. The effective lipopeptide concentration required to obtain 50% growth inhibition of tested fungi (EC_{50}) was calculated by regressing inhibition of fungal growth against the logarithm of LP concentration. Commercial surfactin (C15, Sigma Aldrich) was used as control (data not show).

Compound 1. Yield: 8.4 mg; colorless amorphous solid; $[\alpha]^{25}_D$ -26.5° ($c = 0.1$ MeOH); IR (KBr) ν_{max} 3413, 2928, 1151, 1045 cm^{-1} ; 1H NMR data, see Table 1; ^{13}C NMR data, see Table 1. HRFAB MS (positive ion): found m/z 1036.6928 $[M+H]^+$; calculated for $C_{53}H_{94}N_7O_{13}$ m/z 1036.6914; FAB MS (positive ion): m/z 1037 $[M+H]^+$, m/z 1059 $[M+Na]^+$, m/z 1075 $[M+K]^+$; ESI MS1/MS2: m/z 1059, 946, 933, 815, 718, 700, 693, 580, 467, 396, 281.

Compound 2. Yield: 5.8 mg; colorless amorphous solid; $[\alpha]^{25}_D$ -25.7° ($c = 0.1$ MeOH); IR (KBr) ν_{max} 3410, 2930, 1150, 1045 cm^{-1} ; 1H NMR data, see Figure 1 and Table 1; ^{13}C NMR data, see Figure 1 and Table 1. HRFAB MS (positive ion): found m/z 1022.6769 $[M+H]^+$; calculated for $C_{52}H_{91}N_7O_{13}$ m/z 1022.6757; FAB MS (positive ion): m/z 1023 $[M+H]^+$, m/z 1045 $[M+Na]^+$, m/z 1061 $[M+K]^+$; ESI MS1/MS2: m/z 1045, 932, 919, 801, 704, 686, 679, 566, 453, 382, 267.

Tables and Figures

Table 1. ^1H and ^{13}C NMR data of **1** and **2** at 500 and 100 MHz in CD_3OD . ^a Overlapped with other signals

	1		2	
position	δ_{C} mult.	δ_{H} mult. (<i>J</i> in Hz)	δ_{C} mult.	δ_{H} mult. (<i>J</i> in Hz)
Glu-1				
NH		7.57 d (2.5)		7.57 d (2.5)
C=O	172.4 s		172.4 s	
α	59.7 d	4.04 bt (5.5)	59.7 d	4.03 bt (5.5)
β	26.9 t	2.11 m	26.9 t	2.11 m
γ	30.1 t	2.22 t	30.1 t	2.21 t
δ	173.3 s		173.3 s	
Val-2				
NH		8.20 d (2.5)		8.20 d (2.5)
C=O	172.7 s		172.7 s	
α	52.2 d	4.42 bd (6.0)	52.2 d	4.43 bd (6.0)
β	24.5 t	1.47 dq (6.0)	24.4 t	1.46 dq (6.0)
γ	21.5 q	0.93 d (6.0)	21.6 q	0.93 d (6.0)
Leu-3				
NH		8.40 d (2.5)		8.40 d (2.5)
C=O	172.6 s		172.6 s	
α	52.1 d	4.43 bt (6.5)	52.2 d	4.44 bt (6.5)
β	41.4 t	1.78 dd (6.5, 7.5)	41.4 t	1.78 dd (6.5, 7.5)
γ	24.4 t	1.49 dq (5.5, 6.0)	24.5 t	1.49 dq (5.5, 6.0)
δ	21.3 q	0.92 d (6.0)	21.6 q	0.92 d (6.0)
Val-4				
NH		7.91 d (2.5)		7.91 d (2.5)
C=O	175.5 s		175.5 s	
α	50.5 d	4.46 bd (6.0)	50.5 d	4.47 bd (6.0)
β	24.4 t	1.49 dq (6.0)	24.4 t	1.49 dq (6.0)
γ	17.9 q	0.90 (d (4.0)	17.8 q	0.89 d (4.0)
Asp-5				
N-H)		7.93 d (2.5)		7.93 d (2.5)
C=O	172.3 s		172.3 s	
α	53.9 d	4.18 t (4.5)	53.9 d	4.18 t (4.5)
β	35.2 t	2.98, 2.85 dd (15.0, 4.5)	35.5 t	2.98, 2.85 dd (15.0, 4.5)
γ	173.2 s		173.3 s	
Leu-6				
N-H		8.43 d (2.5)		8.43 d (2.5)
C=O	172.0 s		172.0 s	
α	53.0 d	4.38 bt (6.5)	53.0 d	4.39 bt (6.5)
β	41.1 t	1.70 dd (6.5, 7.5)	41.4 t	1.70 dd (6.5, 7.5)
γ	24.4 t	1.59 dq (5.5, 6.0)	24.3 t	1.59 dq (5.5, 6.0)
δ	21.6 q	0.91 d (6.0)	21.5 q	0.90 d (6.0)
Leu-7				
N-H		8.51 d (2.5)		8.50 d (2.5)
C=O	172.1 s		172.1 s	
α	52.0 d	4.24 bt (6.5)	52.0 d	4.25 bt (6.5)
β	41.0 t	1.71 dd (6.5, 7.5)	41.1 t	1.72 dd (6.5, 7.5)
γ	24.8 t	1.59 dq (5.5, 6.0)	24.9 t	1.59 dq (5.5, 6.0)
δ	21.8 q	0.92 d (6.0)	21.8 q	0.92 d (6.0)
β -OH acid				
CO ₂ H	175.0 s		175.0 s	
α	39.5 t	2.52, 2.46 dd (15.0, 5.0)	39.5 t	2.50, 2.47 dd (15.0, 5.0)
β	72.2 d	5.30 m	72.2 d	5.30 m
γ	33.5 t	2.50, 1.75 m	33.4 t	2.50, 1.74 m
δ	28.9 t	2.63, 2.01 m	28.9 t	2.64, 2.00 m
ε - ω_4	29.6 t	1.30 m	29.6 t	1.30 m
ω_3	31.9 t	1.34 m ^a	31.9 t	1.33 m ^a
ω_2	22.7 t	1.34 m ^a	22.7 t	1.33 m ^a
ω	16.0 q	0.92 t	16.0 q	0.92 t

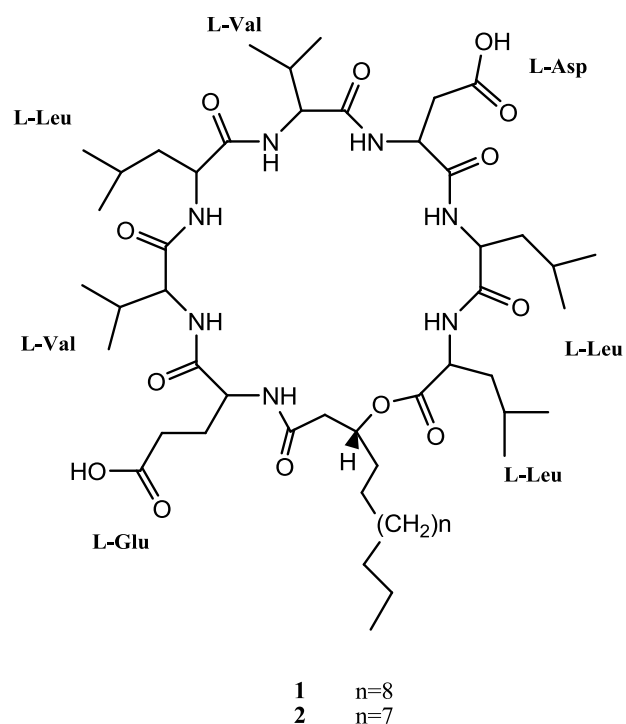
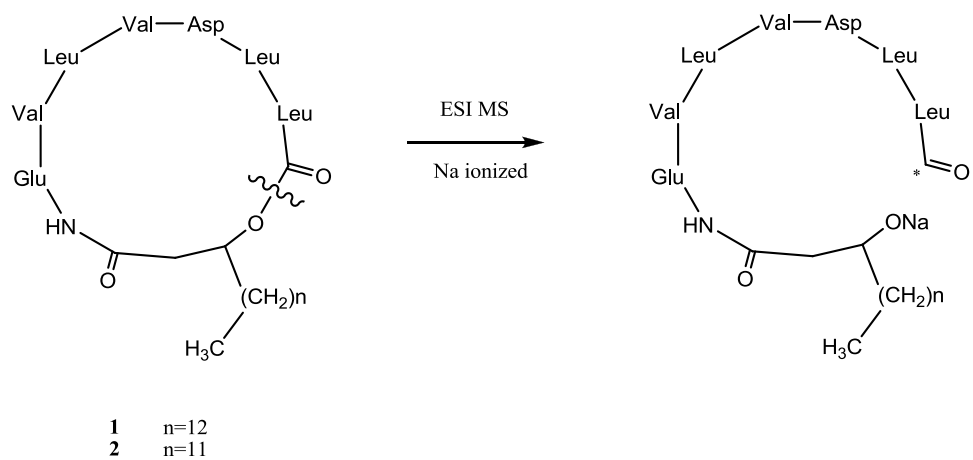


Figure 1. Structure of compounds 1 and 2 purified from *Bacillus subtilis* strain BO5A

(A)



(B)

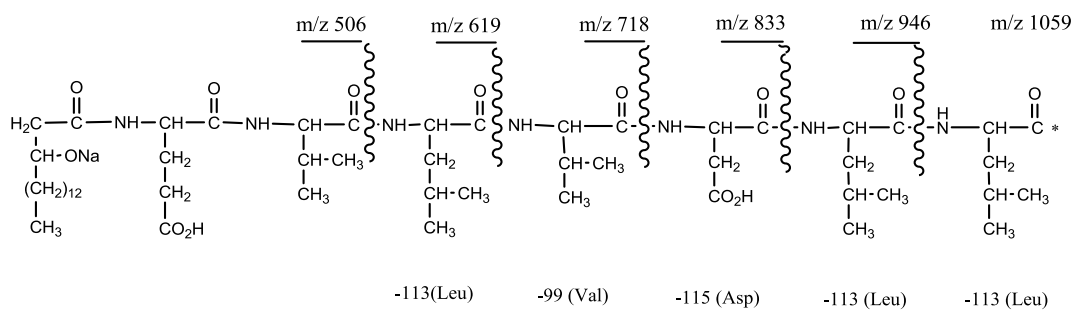
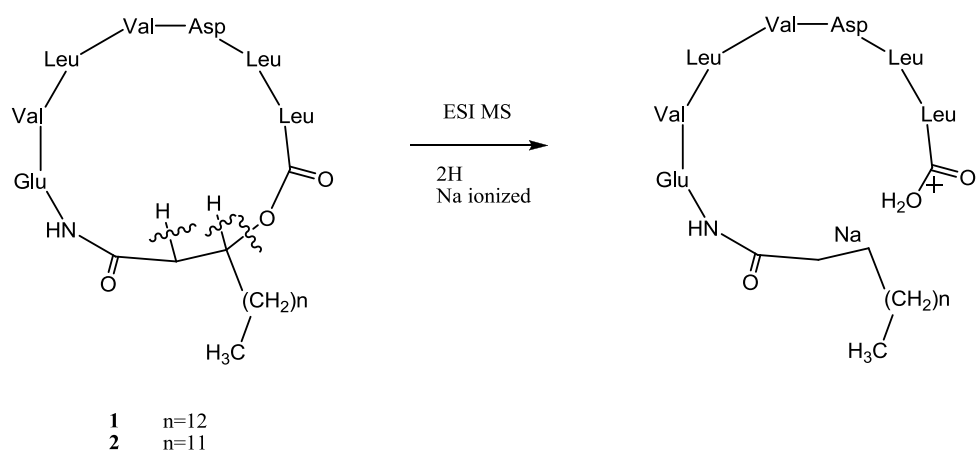
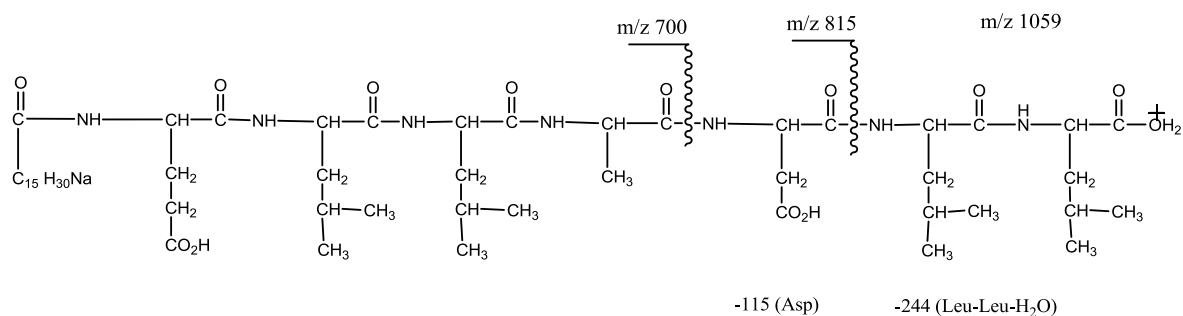


Figure 2. (A) Simple cleavage of 1 and 2 in MS. (B) A set of fragmentation ions obtained in tandem MS of compound 1

(A)



(B)



(C)

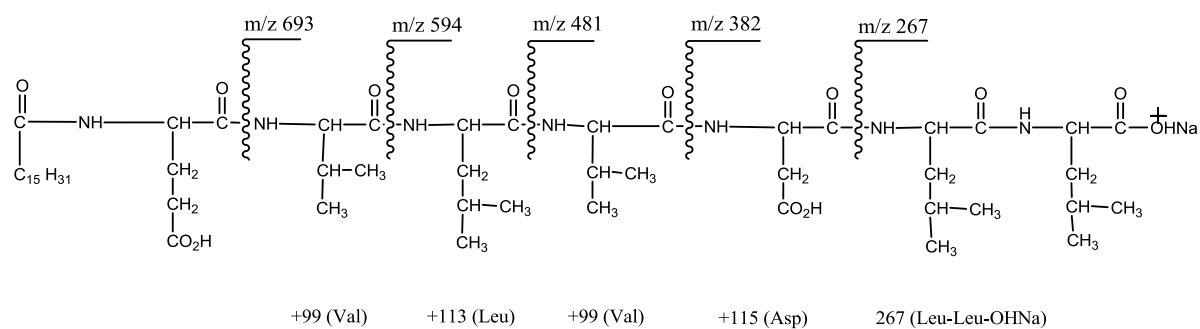


Figure 3. (A) Further cleavage of 1 and 2 in tandem MS. (B) Molecular and fragmentation ions obtained in tandem MS of compound 1. These ion peaks are 18 amu higher of corresponding peaks after simple cleavage. (C) A set of fragmentation ions obtained in tandem MS of compound 1

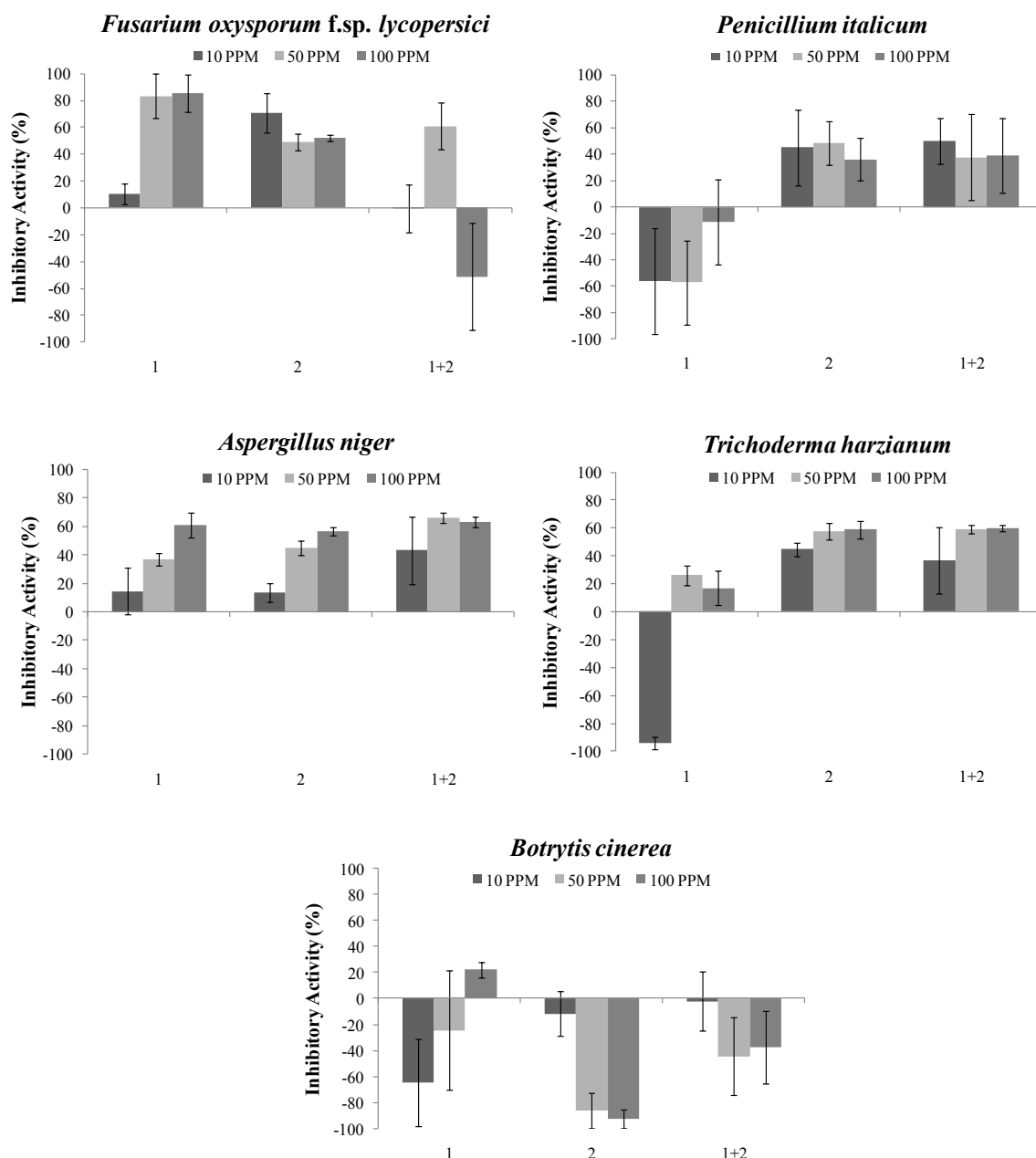


Figure 4. Antifungal activity of compounds 1 and 2 against five common fungal pathogens. Each compound, either alone or in combination, was tested at the final concentrations of 10, 50 and 100 ppm, using a bioassay in microtiter plates. Fungal growth was measured spectrophotometrically at 595 nm after 72 h incubation and inhibitory activity (IA) was calculated as the percentage of fungal biomass reduction compared to the control (EtOH =0%). Bars indicate the standard deviation from 4 replicates

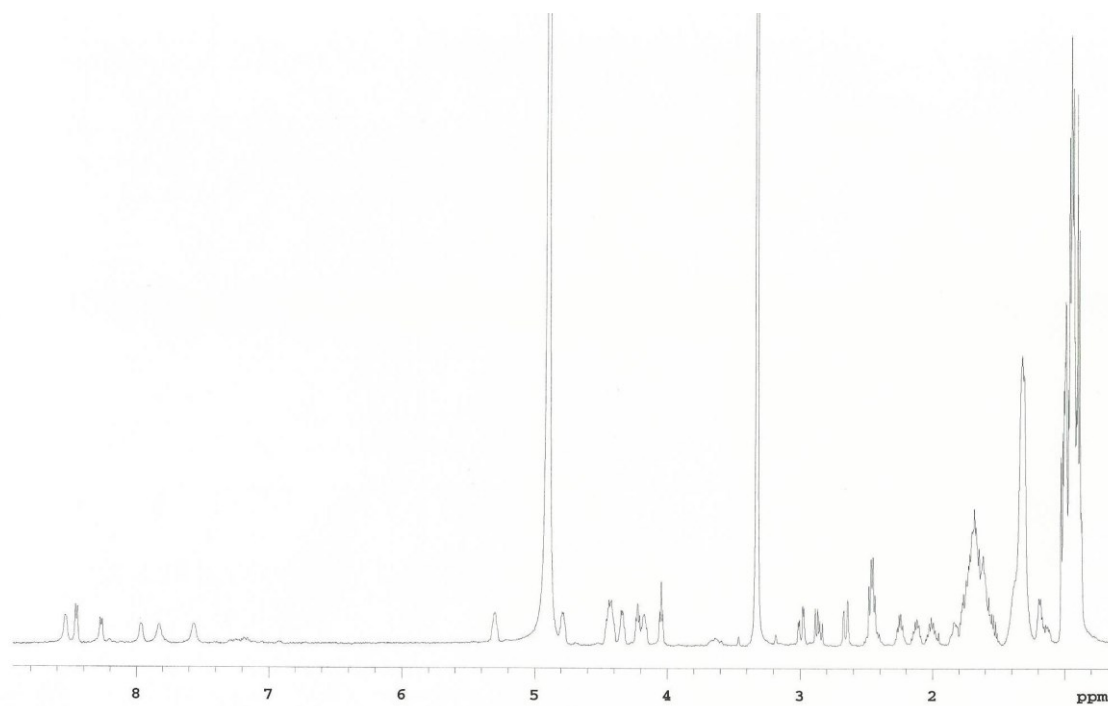


Figure S1. ^1H NMR spectrum (CD_3OD) of compound 1

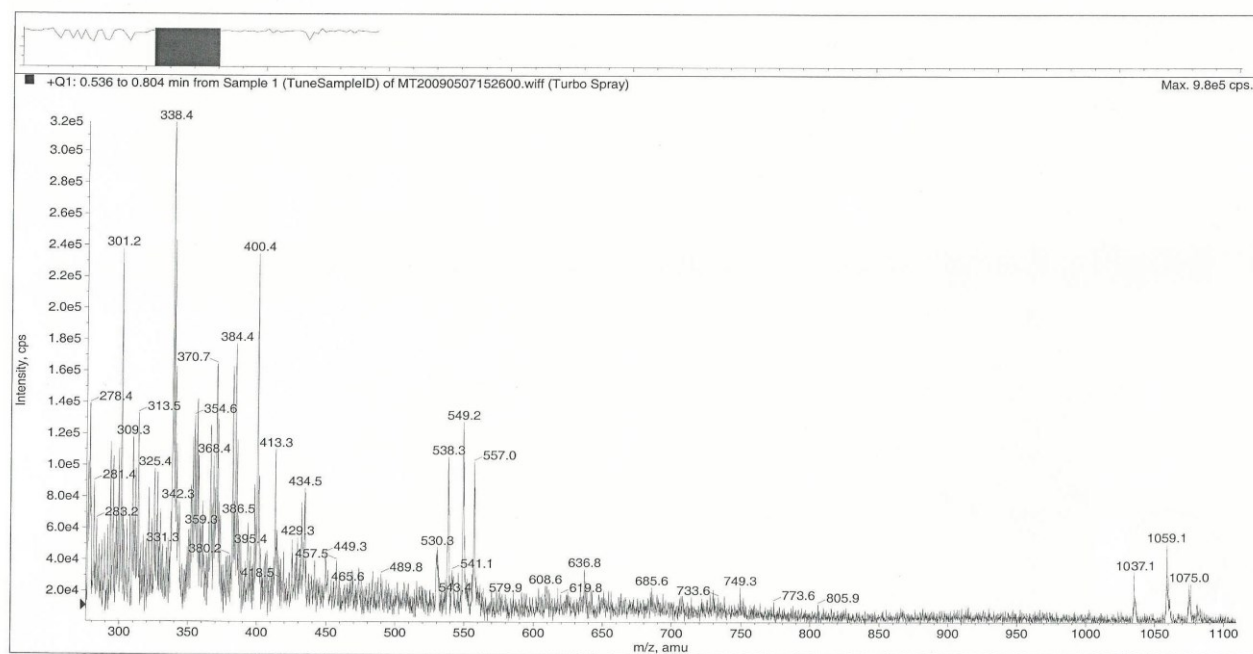


Figure S2. MS of compound 1

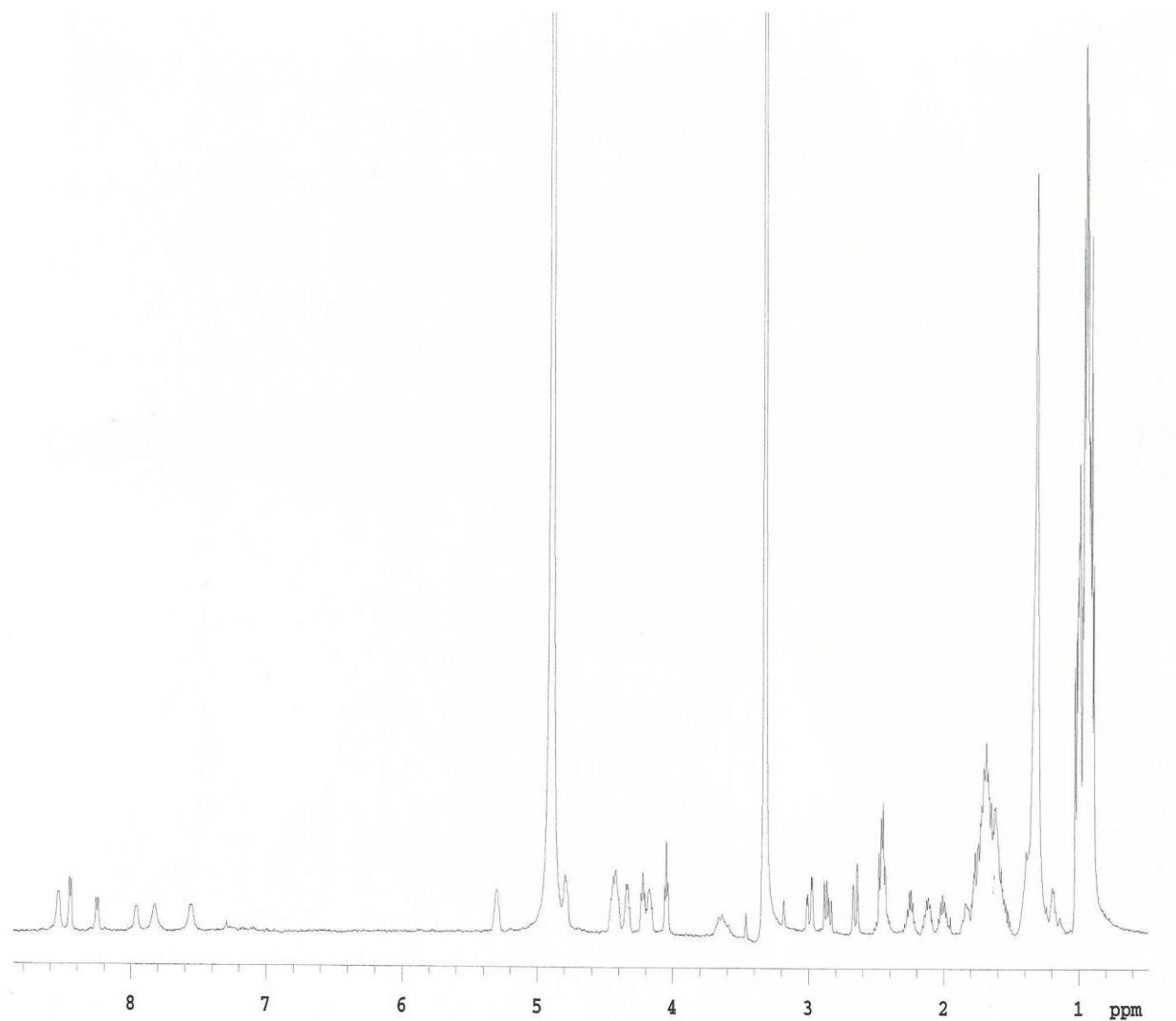


Figure S3. ¹H NMR spectrum (CD₃OD) of compound 2

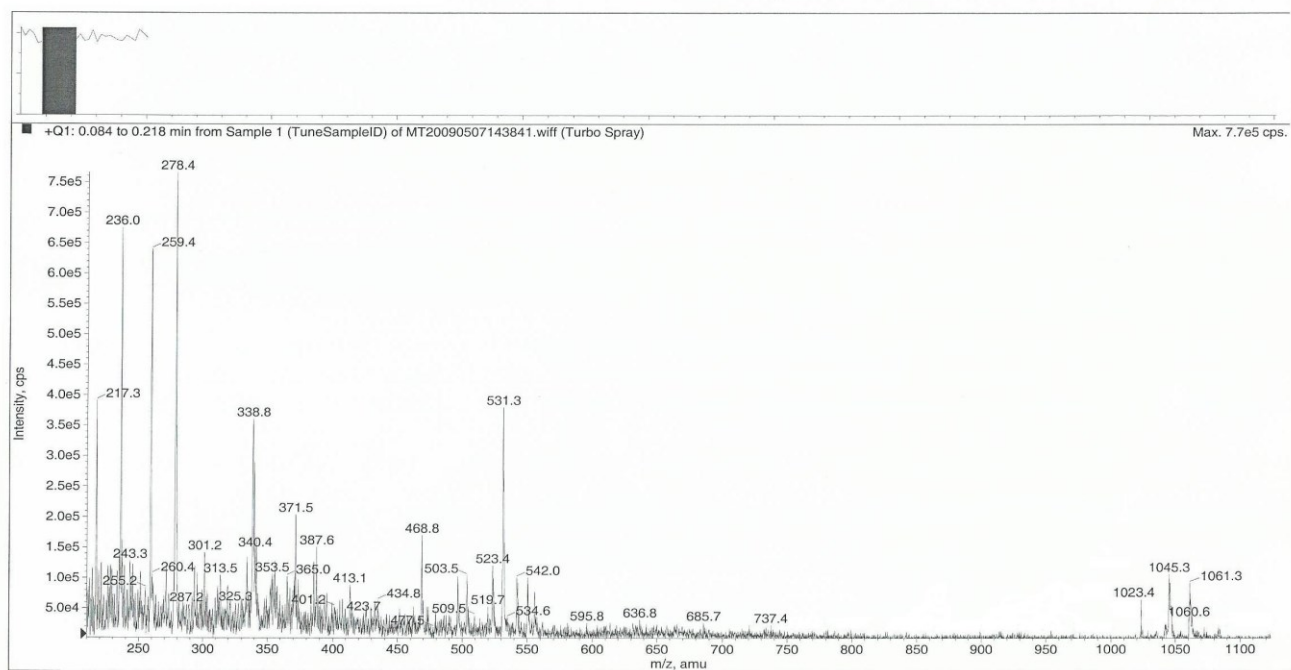


Figure S4. MS of compound 2

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Chapter 2: Toxicity of self-DNA in litter and plant-soil negative feedback

Summary

- Plant-soil negative feedback (NF) and litter autotoxicity are recognized as important factors shaping natural plant communities. Objectives of this work were to: (i) assess the effects on root proliferation of litter phytotoxicity and autotoxicity; (ii) test the hypothesis of self-DNA driving litter autotoxicity and plant-soil NF.

- A litterbag experiment was carried-out for 120 days with 20 litter types. Litter inhibitory effects were studied by bioassays with eight target species. Activated carbon (AC) was used to assess the relative contribution of different toxic factors. DNA accumulation was measured in litter and soil history experiments. Finally, self-DNA toxicity was tested in laboratory bioassays.

- Our results demonstrated that undecomposed litter causes strong nonspecific inhibition of root growth while species-specific autotoxicity was recorded for aged plant litter. AC addition removed fresh litter phytotoxicity, but was ineffective against autotoxicity of aged materials.

- Phytotoxicity was found to be related to known allelopathic compounds rapidly disappearing during litter decomposition. Significant DNA accumulation was observed in both litter decomposition and soil history experiments. DNA showed evident species-specific inhibitory effects.

- Our results indicate a general occurrence of litter autotoxicity, identifying self-DNA as responsible for the observed inhibitory effects. Reported evidence also suggests the involvement of extracellular DNA in plant-soil NF.

Introduction

Negative plant-soil feedback (NF), i.e. the rise in soil of negative conditions for plants induced by the plants themselves (Klironomos, 2002), is recognized as an important factor shaping natural plant communities (van der Putten et al., 1993) and allowing species coexistence (Bever et al., 1997, Bonanomi et al., 2005). NF has been observed in a variety of environments including grasslands (Reynolds et al., 2003), temperate and tropical forests (Mangan et al., 2010) and also anthropogenic agroecosystems (Singh et al., 1999).

The large body of available data in the literature on species-specific NF (Kulmatisky et al., 2008; van der Putten et al., 2013) has been related to different non-mutually exclusive hypotheses of possible underlying mechanisms: soil nutrient depletion (Ehrenfeld et al., 2005) and the build-up (Packer and Clay, 2000) and changing composition (Kardol et al., 2007) of soil-borne pathogen populations. However, observations of long-term “soil sickness” and “replant diseases” in agriculture (Zucconi, 1993, 2003) show examples of species-specific inhibition unrelated to either nutrient availability or soil-borne pathogens and similar findings are also reported for natural ecosystems (Bonanomi et al., 2011a).

Another hypothesis proposed the release of phytotoxic compounds during litter and organic matter decomposition as a further possible mechanism to explain NF (Singh et al., 1999). This has been based on the observation that, in addition to the well recognized role of litter as a nutrient source (Vitousek and Sanford, 1986), leaf and root litter can also inhibit plant growth by immobilizing nitrogen (Hodge et al., 2000) or by releasing phytotoxic compounds during decomposition (An et al., 2001; Trifonova et al., 2008). However, such toxins are known to be rapidly degraded by soil microbial activity. In fact, litter inhibitory effects have been found to be limited to short-term phases of early decomposition stages, usually lasting only few weeks (Bonanomi et al., 2011b; Hodge, 2004). For this reason, this hypothesis has been widely criticized (Fitter, 2003; Harper, 2010). Indeed, in traditional studies of litter allelopathy, hundreds of organic compounds, extracted from plant tissues, purified and identified (e.g. Rice, 1984; Rizvi and Rizvi, 1992; Reigosa et al., 2006), only showed a general toxicity without species-specific effects. It seems evident that such phytotoxic compounds, provided their lack of specificity and short persistence in the soil, can hardly explain species-specific plant-soil NF.

In this context, it is important to note that plant-soil NF mainly occurs in terrestrial systems while is rarely found in aquatic environments (Mazzoleni et al., 2007). Consistently with such indirect observations, modelling work demonstrated how the release of inhibitory factors by litter decomposition and their removal by water can explain the latitudinal gradient of plant species diversity as well as the co-occurrence of monospecific stands of mangroves and riparian vegetation with highly diverse tropical forests (Mazzoleni et al., 2010). Recently, the accumulation of autotoxicity in soil was also proposed as a driver of the spatial organization of vegetation (Carteni et al., 2012), with water dynamics affecting plant-soil NF (Marasco et al., 2013).

Based on the aforementioned considerations, in the search for a molecule responsible for plant-soil NF and litter autotoxicity we considered three main clues: i) long-lasting persistence in the environment; ii) specificity of effect and iii) water solubility. All such indications pointed out extracellular DNA as the target of our hypothesis.

In this work we investigated the frequency of occurrence of litter autotoxicity and the involvement of self-DNA toxicity as a putative underlying mechanism.

Specific hypotheses tested in this work were: i) plant litter exerts species-specific inhibitory effects (i.e. autotoxicity); ii) biochemical litter quality changes during decomposition, including DNA accumulation; iii) purified plant DNA exerts size-dependent (i.e. toxicity decrease with molecular weight) and species-specific (i.e. involve mainly conspecifics) inhibitory effects. Moreover, to assess the relevance of self-DNA toxicity under real ecological conditions and its association with NF, two complementary approaches were used: i) evaluation of growth of a test plant on different soil types treated with self-DNA; ii) a soil history experiment (Klironomos, 2002) aimed to assess the accumulation of plant DNA in soil with different soil histories and its relation with NF effects.

Materials and Methods

Litter collection and decomposition experiment

Twenty different plant species were selected from vegetation types of both Mediterranean and temperate environments. The species pool, representing a wide range of litter quality, included: one annual herb (*Lepidium sativum*), four perennial grasses (*Acanthus mollis*, *Ampelodesmos mauritanicus*, *Festuca drymeia* and *Medicago sativa*), two evergreen shrubs (*Arbutus unedo*, *Coronilla emerus*), one vine (*Hedera helix*), four evergreen trees (*Cupressus sempervirens*, *Picea excelsa*, *Pinus halepensis*, *Quercus ilex*), and eight deciduous trees (*Alnus cordata*, *Castanea sativa*, *Fagus sylvatica*, *Fraxinus ornus*, *Populus nigra*, *Quercus pubescens*, *Robinia pseudoacacia*, *Salix alba*). Freshly abscised leaves were collected by placing nets under plants of natural communities (in the case of grasses, standing senescent leaves were cut and collected - n° plants >20 randomly selected individuals for each species), dried at room temperature in a ventilated chamber until constant weight was reached and then stored at room temperature.

Decomposition experiments were carried out in microcosms placed in a growth chamber to simulate field decomposition conditions. Under field conditions, litter decomposition depends on organic matter quality, water availability and temperature (Gholz et al., 2000; Berg and McClaugherty, 2008). In this experiment, in order to focus only on litter quality effects, controlled conditions of optimal water availability and temperature were used. Dry leaf litter (100 g for each species per replicate) was placed inside plastic trays (sized 30 x 50 x 50 cm). For each plant species, three replicate trays were used. A microbial inoculum was prepared and distributed according to Bonanomi et al. (2011b). Controlled environmental conditions were: temperature $18\pm 2^{\circ}\text{C}$ night and $24\pm 2^{\circ}\text{C}$ day, watered every seven days to holding capacity with distilled water.

Trays were harvested after 0, 30 and 120 days of decomposition for a total of 180 samples (20 species x 3 sampling dates x 3 replicates). Collected litter was dried in paper bags in the laboratory (40°C until constant weight was reached) and the remaining material weighed. In this way we produced 60 organic materials (20 species at three sampling dates) with different ages: fresh undecomposed litter (thereafter indicated as 0 days) and decomposed litter for 30 and 120 days.

Assessment of litter effects on target plant species

Bioassays were conducted in root observation plate (Mahall and Callaway, 1992) to assess the capability of plant seedling roots to colonize different litter types. Activated carbon (AC) was applied to evaluate the relative toxicity and N immobilization effects as causes of observed growth inhibition (Mahall and Callaway, 1992; Hille and den Ouden, 2005). In order to determine if litter had species specific effects, we tested the 20 litter types at different decomposition ages (total of 60 different substrates) on 8 target plants. These were selected as a subset of the 20 species of the decomposition experiment, including species representatives of herbaceous, shrub and tree growth forms. Such experimental design enabled to compare the litter effects on conspecific and heterospecific plants, i.e. the autotoxicity and phytotoxicity effects, respectively.

The bioassay mostly followed the methods described in a previous paper (Bonanomi et al., 2011b). Briefly, a 8 cm wide sterile filter paper strip was placed in square Petri dishes (size 12 x 12 x 1.5 cm). Pre germinated seeds of each species (5 for each dish) were placed at the top of the paper strip that was previously amended with AC and different litter types. Larger root observation plates (30 x 20 x 1.5 cm) were used for target plants with large sized seeds (*Pinus halepensis* and *Quercus ilex*). Root observation

plates were oriented with a 45° slope on a horizontal surface so that the positive geotropism of roots would allow a growth down along the plate. Plates were covered with opaque sheets when roots were not under observation.

The experimental design included two treatments: 1. control with sterile distilled water; 2. addition of AC (Sigma-Aldrich Co.) at 0.2 g/cm² of filter paper. These two treatments were applied with and without litter (Fig. 5). Dry, powdered litter (separately, each of the 20 species at 3 decomposition stages for a total of 60 samples) was applied at the rate of 0.2 g/cm² over filter paper. Experimental values of litter addition were set to be within the range of observed litterfall of natural ecosystems (Vogt et al., 1986). Each treatment was replicated 5 times. Globally, 4,800 experimental units (root observation plates) have been made (60 litter types x 2 treatments x 8 target plant species x 5 replicates) for a total of 24,000 seedlings analyzed. Seedling root length was measured after a variable number of days since the start of the experiment depending on the target species. Petri dishes were arranged in a growth chamber, according to a totally randomized design, under controlled environmental conditions (temperature 22±2°C, watered every two days with distilled water). For each litter material, growth recovery due to AC addition was calculated as the difference of root length between treated and untreated plates, and expressed as percentage of the latter.

Extraction and quantification of DNA from plant materials

DNA was extracted from dried litter materials by using a - DNeasy Plant Mini Kit as described by the manufacturer (QIAGEN, Valencia, CA). 100 mg dry weight of starting material was eluted in 60 µl of sterile water. DNA extracts were spectrophotometrically quantified at 260 nm on NanoDrop TM 1000 (Thermo scientific, Wilmington, DE) and visually verified on 1.5% agarose gel by Sybr[®] Safe (Invitrogen).

For plant leaves, MAXI DNA extraction was performed to prepare the treatment solution using the protocol by Fulton et al. (1995) with some modifications. 100 grams fresh weight of starting material were harvested and frozen at -20°C. Then, the samples were blended immersed in 200 ml fresh microprep buffer, then filtered in cheese cloth. DNA was resuspended in adequate pure sterile water. The extracted DNA from leaves was fragmented by sonication. This was performed by a Bandelin Sonopulse (Bandelin, Berlin, DE) at 90% power with a 0.9 seconds pulse for 12 minutes. Verification of sonicated bands size was performed visually verified on 3% MetaPhorTM agarose gel (Lonza scientific, NJ) by Sybr[®] Safe (Invitrogen).

Effects of purified DNA on target plants

A set of bioassays were done to assess both autotoxic and phytotoxic effects of purified DNA extracted from plant leaves. The target plants included seven species whose litter was used in the litter toxicity experiment with the addition of *Arabidopsis thaliana* used as a standard model plant. Autotoxicity was assessed by using DNA extracted from the leaves of each target species, instead phytotoxicity was tested using a mixture of DNA extracted from heterospecific species. In detail, the mixture was composed by equal amounts of DNA from seven of the heterospecific species. The DNA was extracted from leaves of all plant species as described in the previous section.

For each target species, the experimental design included the following treatments: 1. control with sterile distilled water; 2. addition of genomic self-DNA; 3. addition of sonicated self-DNA; 4. addition of a mixture of genomic DNA from heterospecific; 5. addition of sonicated mixture of heterospecific DNA. 6. addition of sonicated DNA from single heterospecific (only in the cases of *A. thaliana*, *A. mollis*, *L. sativum*). DNA was applied in both genomic and fragmented forms to represent its different decay levels observed during the decomposition process. The fragmentation of DNA was done by sonication as reported above. In each treatment, DNA has been applied at three different concentrations (200, 20, and 2 $\mu\text{g ml}^{-1}$). The bioassays were done in vitro by using surface sterile seeds ($n=20$ in each plate) for each species placed in 9 cm Petri dishes over sterile filter papers imbibed with 4 ml of test solutions. Each treatment has been replicated 10 times. Seedling root length was measured after a variable number of days since the start of the experiment depending on the target species. Petri dishes were arranged in a growth chamber, according to a totally randomized design, under controlled environmental conditions (temperature $22\pm 2^\circ\text{C}$, watered every two days with distilled water).

DNA autotoxicity was also assessed in a greenhouse experiment with two soil of contrasting texture (sandy and clay). DNA was extracted from the leaves of the target species *Medicago sativa*. We selected *M. sativa* because such species is the most important forage plant worldwide, is a model plant and in a previous study we demonstrate that suffers a strong NF related to litter autotoxicity (Bonanomi et al., 2011b). The DNA was extracted from fresh leaves as described in the previous section. The experimental design included the following treatments: 1. control with sterile distilled water; 2. addition of genomic self-DNA; 3. addition of sonicated self-DNA. DNA has been applied one time at the beginning of the experiment with solution at three

different concentrations (2000, 200, and 20 $\mu\text{g ml}^{-1}$) until soil reached field capacity. The bioassays were done in greenhouse by using seeds ($n=5$ in each pot) placed in 12 cm diameter pots. Two contrasting soil textures were selected to preliminarily evaluate their impact on DNA toxicity. In detail, the first was a clay soil (sand 45%, silt 21.5 %, clay 33.5%, pH 8.21, organic matter 0.85%, total N 0.81 g/kg, C/N 6.1, total CaCO_3 189 g/kg, available phosphorus (P_2O_5) 6.2 mg/kg, exchangeable potassium 0.46 meq/100 g, exchangeable magnesium 1.89 meq/100 g, exchangeable calcium 27.6 meq/100 g, exchangeable sodium 0.35 meq/100 g, EC 0.096 dS/m) and the second was a sandy soil (sand 83.9%, silt 1.9 %, clay 14.2%, pH 8.41, organic matter 0.55%, total N 0.65 g/kg, C/N 5, total CaCO_3 140 g/kg, available phosphorus (P_2O_5) 48.1 mg/kg, exchangeable potassium 0.53 meq/100 g, exchangeable magnesium 0.96 meq/100 g, exchangeable calcium 15.5 meq/100 g, exchangeable sodium 0.17 meq/100 g, EC 0.188 dS/m) (Tab. 1). Each treatment has been replicated 20 times for a total 120 pots. Plant shoot and root dry biomass was measured after 60 growing days since the start of the experiment. Pots were arranged in a greenhouse, according to a totally randomized design, under controlled environmental conditions (temperature $22\pm 2^\circ\text{C}$, watered every two days with distilled water).

Plant growth in vitro was statistically analyzed considering DNA origin (conspecific versus heterospecific), fragmentation levels (genomic versus sonicated), and concentrations (three levels) as independent factors. For each target species and for all species pooled, differences of root growth related to DNA origin at each concentration level, or dependent on concentration within each DNA source, were tested for statistical significance using Mann-Whitney-Wilcoxon test. In the greenhouse experiment, two-way ANOVA and post-hoc Duncan test were used to assess the effect of DNA treatment and soil type on plant growth.

Soil history experiment

In the context of plant-soil feedback study, the soil history experiment represent a widely used approach to investigate the effects and the mechanisms underlying NF. This method, although based on a simplified rooting environment, is widely accepted (Klironomos, 2002; Kulmatisky et al., 2008; Petermann et al., 2008). It allows the investigation of the soil heterogeneity induced by plant root systems without the confounding factors of above ground litter and other root systems. Here, two main questions have been addressed by this experiment: i. does plant growth build-up a plant-

soil negative feedback in a soil collected in the field? ii. are changes of plant DNA accumulation associated with the observed plant-soil feedback? *Medicago sativa* was selected for the production of the soil history for the reason described in the previous section. A sandy agricultural soil (see previous section for full description) was collected in the field (~100 kg) from the first 20 cm depth after litter removal at the surface. The following day, the soil was sieved at the laboratory (mesh size < 2 mm). Pots (18 cm of diameter, 25 cm of height) were filled with 1000 g of a mixture of dry soil and perlite (4:1 v/v). Three different soil treatments were settled by growing *M. sativa* as mono-cultures for two different periods and using the bare soil as control. Seeds were sown in the pots (10 seeds per pot). After 21 days, germinated seedlings were thinned at a density of three plants per pot. Plants were daily watered with distilled water at field capacity without addition of nutrient solution. Pots were weeded weekly to avoid the emergence of seedlings of other species from the soil seed bank. In a first treatment irrigation was maintained for 360 days and then stopped to dry the soil until plant death which occurred within 15 days. A second treatment was started later in the year and lasted for 180 days. At the end of treatment, dry above-ground biomass was removed by cut at soil surface without any soil disturbance. The pots containing the soil and the residual dead roots were used to establish the growth experiments. In the second experimental phase in all soil types (three soil histories) three *M. sativa* individual plants were grown in each soil type. The experiment was organised according to a completely randomised factorial design with a target species and three soil histories, each with 20 replicates for a total of 60 pots (3 x 20). Pots distribution was rearranged at random within the greenhouse every week. Seedlings were obtained from germinated seeds in 9-cm petri dishes with wet filter paper in growth chamber at +25 °C. Seedlings were transplanted into 1 cm deep holes in a triangular design into the pot. Withered seedlings were replaced within the first 48 hours. Plants were daily irrigated with distilled water without any nutritive solution. All the experiment was conducted in a growth chamber in springs 2012 with temperature ranging from 20 to 28 °C with natural day length. During the experiment plant mortality in each soil history was monitored after 10, 30, 90 and 180 days from the beginning of the experiment. At the end of the experiment, after 180 growing days, above ground dry biomasses of living plants were measured after desiccation at +105 °C for 72 hours. Before the start of the second experimental phase, 5 g of soil were taken from pots for each soil history and DNA content was measured as below described. Statistical analysis was done by one-way ANOVA on log transformed data, required for normalization.

Total genomic DNA from soil was isolated from raw samples of 100 mg each, resuspended in cTAB extraction buffer (Tris-HCl pH 7.5, 0.1 M; NaCl 0.5 M; EDTA 0.05 M; CTAB 1%) and mixed with stainless steel beads. Samples were vortexed for 2 minutes and put at 65°C for 1 hour with occasional shaking, then centrifuged at room temperature for 5 minutes at max speed. Supernatant was transferred to new tube and mixed with 1 volume of phenol:chloroform:isoamyl alcohol 25:24:1 then centrifuged at max speed for 2 minutes and supernatant was transferred to a new tube and mixed with 1 volume of chlorophorm:isoamyl alcohol 24:1. Last step was repeated two times. Finally supernatant was transferred to a new tube and DNA was precipitated with one volume of ice cold isopropanol, centrifuged for 5 minutes at max speed and washed with 70% ethanol, air-dried and resuspended in double-distilled sterile water. Purified DNA was quantified spectrophotometrically at Nano-drop (Thermo scientific) (all with 260:280 ratios above 1.7) and by comparison to DNA standards using agarose gel electrophoresis.

Results

Litter effects in laboratory conditions

In the first experiment we observed that plant litter has a species-specific effect on plant growth. The observed inhibition of root growth depends on litter type and age, plant species and active carbon addition (AC) (Fig. 1a,b). All undecomposed litter materials showed an inhibitory effect on *Lepidium sativum*, a reference plant commonly used in bioassay tests (Fig. 1a). Such inhibition largely varied according to litter type, being very high in the case of litter from *Coronilla emerus*, *Acanthus mollis*, *Medicago sativa*, *Pinus halepensis*, and *Hedera helix*, relatively low for grasses (*Festuca drymeia* and *Ampelodesmos mauritanicus*) and most broadleaved deciduous trees (*Salix alba*, *Castanea sativa*, *Fagus sylvatica*, *Populus nigra* and *Quercus pubescens*), and intermediate for the remaining species (Fig. 1a). The test plant *L. sativum* was also strongly inhibited by its own litter. Decomposition for 120 days greatly reduced the inhibitory effect with the only exception of *A. mollis* (Fig. 1b). It should be noted, however, that *Lepidium* autotoxicity (i.e. growth inhibition on conspecific litter) was still very high, also in the case of decomposed litter.

The phytotoxic and autotoxic effect was further tested on eight different target species selected among the litter types and representative of different functional groups (i.e. herbaceous grass and forbs, as well as evergreen and deciduous woody species). Root

growth of target species was significantly inhibited on undecomposed heterospecific litter, while it showed a better performance with increasing litter age (Fig. 1c). Active carbon (AC) application showed a highly significant interaction with litter age. Highly significant root growth recovery was observed after AC addition on undecomposed litter, whereas progressively decreasing recovery by AC addition was found with increasing litter age (Fig. 1e). Opposite dynamics to those observed on heterospecific materials were observed when the analyses were restricted to conspecifics. In this case root growth was inhibited by the aged litter materials (Fig. 1d) and application of AC did not produce any significant effect on growth recovery (Fig. 1f). The opposite effects of heterospecific and conspecific litters were consistent for all target species (Fig. 2), showing a general common pattern of species-specific inhibition.

DNA quantification on plant litter

Direct plant litter DNA quantification by agarose gel electrophoresis showed a progressive increase of DNA concentration with decomposition time (Fig. 3a,b). An evident decrease of the size of the DNA molecules was also observed in most litter materials (Fig. 3c). In details, only polynucleotides of less than 500 base pairs were found in litter decomposed for 120 days. The increase of DNA content with litter age was also indirectly supported by the significant positive correlation with the variation of NMR signals resonating at δ 150-154 from the same materials. The only exception to such trend was the case of *Pinus* and *Picea*, with higher DNA concentrations in the undecomposed materials, suggesting a rapid degradation of DNA with litter age in these coniferous species. Further investigations are required to understand the reasons for this degradation pattern which might be related to the acidic conditions of these litter types.

Effects of DNA on target species

In the first experiments, DNA from conspecific and heterospecific were supplied in two forms, either as obtained by genomic DNA extraction or as fragments produced by sonication of the extracted DNA. The root growth of all nine target species was significantly inhibited by sonicated self-DNA (Fig. 4a), in a concentration dependent way (Fig. 4b), while the mixtures of sonicated heterologous DNA, as well as genomic self-DNA (data not shown), did not affect root growth (Fig. 4a).

In the second experiment where DNA was added to two different soils, purified self-DNA inhibited *M. sativa* seedling growth in a concentration dependent manner.

Growth was inhibited at the middle and highest concentration (200 and 2000 p.p.m.) in both soil types, with a more evident harmful effect in sand compared to clay soils (Fig. 7).

Soil history experiment

In the soil history experiment *M. sativa* plants showed a NF proportional to the duration of the previous growth period of the same species on the soil substrate (growth inhibition, compared to bare soil control, was 22 ± 4 % and 35 ± 6 % in soils that had supported growth of *M. sativa* for 180 and 360 days, respectively). The NF was associated with different accumulation of DNA in the soil (135 ± 25 ng mg⁻¹ and 240 ± 12 ng mg⁻¹ in short and long previous growth duration, respectively).

Discussion

Litter phytotoxicity and autotoxicity

In our experiment all undecomposed litter caused a strong inhibition of root growth of the eight target species, thus confirming the findings of previous studies (Bonanomi et al., 2006; Bonanomi et al., 2011b). Thus, a consistent body of evidence indicate that the inhibitory effects of undecomposed litter is a rather general phenomenon not restricted to a few “allelopathic” plants. The initial phase of decomposition basically consists of plant tissue breakdown and subsequent release of cell contents. At this stage, litter inhibitory effects has been related by early studies to the occurrence in plant tissues of several toxic, allelopathic compounds (e.g. Rice, 1984), mainly phenols and aromatics of low molecular weight. Such compounds show a rapid turnover during the decomposition process, with consequent disappearance of their observed allelopathic effect. In this regard, the significant recovery after AC addition of plant growth over undecomposed litter indirectly supports the phytotoxic hypothesis because AC easily absorb low molecular weight compounds. Here, the recovery of root proliferation over fresh litter was almost complete for all species supporting the finding of previous studies (Nilsson, 1994; Hille and den Ouden, 2005). As alternative explanation, some studies reported N immobilization as a mechanism explaining litter inhibitory effects (Hodge, 2004). In our case, and consistently with previous findings (Bonanomi et al., 2011b), litter C-to-N ratio and N release during decomposition were unrelated to root growth inhibition, thus excluding N limitation as a possible causal factor for the observed root growth inhibition at least in the tested species (Fig. 6).

Differently from fresh litter, the inhibitory effects of aged plant residues was species-specific. Decomposition for 120 days greatly reduced and in several cases annulled the inhibitory effect on heterospecific target plants. In contrast, root growth was highly inhibited only by aged litter derived from the same species. In addition, application of AC did not produce any significant growth recovery of target species growing over they own aged litter (Fig. 1f). These results, achieved for all eight tested species, show a common pattern of species-specific, autotoxic inhibition for aged plant litter. In conclusion the fact that the inhibitory is species-specific (i.e. affects mainly conspecific), long-lasting (occur for aged litter) and not affected by AC application suggest that the underlying toxic compound/s could have a different nature compared to compound/s present in fresh litter.

DNA accumulation, litter autotoxicity, and plant-soil NF

Our experimental evidence from the bioassays, although indirect, indicate that autotoxicity can be related to water soluble, stable and large-sized compounds, since they are persistent in the old decayed litters and are not affected by AC addition. Significant DNA accumulation in litter and soil has been previously reported (e.g. Dilly et al., 2004). Indeed, DNA molecular properties allows its persistence in the environment differently from the reported cases of labile allelopathic compounds. This is coherent with the persistence of NF in soils for months and even years, observed in both natural vegetation (Hawkes et al., 2013) and agro-ecosystems (Miller, 1996). Large amounts of extracellular DNA have been reported to occur both in soil and marine sediments (Steffan et al., 1988). DNA is able to persist in soil because of its chemical stability and its protection against enzymatic degradation by absorption and binding onto soil minerals and organic matter components (Levy-Booth et al., 2007). This is also supported by reports on traces of DNA from transgenic plant sequences in soil, even when the presence of plant tissues is no longer detectable (Dale et al., 2002). Noteworthy, extracellular DNA is not preserved in its original genomic size, but it is fragmented in a broad range of dimensional classes, from hundreds to some thousands base pairs in length, as we observed in decaying litter. At this stage, however, an undeniable caveat of our work is related to the origin of DNA. In detail, we quantified total DNA in litter and soil systems that derive from both decomposing plant tissues as well as from microbial turnover (Levy-Booth et al., 2007). Because our hypothesis state that only self-DNA is inhibitory, future studies can deserve special attention in the quantification of self-DNA in litter and soil to effectively quantify

the amount present in natural environments. The known capability of plant root cells to uptake extracellular DNA (Paungfoo-Lonhienne et al., 2010), together with the discovery here reported that self-DNA can be toxic indicate that future studies can consider self-DNA toxicity as a further mechanism to be taken into account to explain species-specific NF. We also suggest that a plant weakening due to the exposure to extracellular self-DNA could eventually increase the susceptibility to pathogens attack. Indeed, future studies will be necessary to clarify the co-occurrence and interactions of these different mechanisms in the development of NF in different environments (van der Putten et al., 2013), including tropical forests where it play a crucial role in maintaining species diversity (Mangan et al., 2010).

Mechanisms behind self-DNA toxicity

Extracellular DNA has been found to serve several biological functions. These include the formation of neutrophil nets as protection from pathogens (Brinkmann et al., 2004), structural components of microbial biofilms (Whitchurch et al., 2002), modulation of plant-pathogen interactions (Wen et al., 2009), source of nutrients for microbes and plants (Paungfoo-Lonhienne et al., 2010), and source for horizontal gene transfer (Doerfler et al., 1995). Our discovery highlights an unexpected additional function of DNA in litter-soil system, where it accumulated and may operate as specie-specific inhibitory compound.

We propose the hypothesis that a mixture of roughly random self-DNA fragments, such as those produced during decomposition of soil organic matter and in our experiment by sonication of genomic DNA, may act either as a multiple “ASO-like” exposure or as sequence specific inhibitors of genome functionality, thus explaining the rise of species-specific inhibitory effects.

Tables and Figures

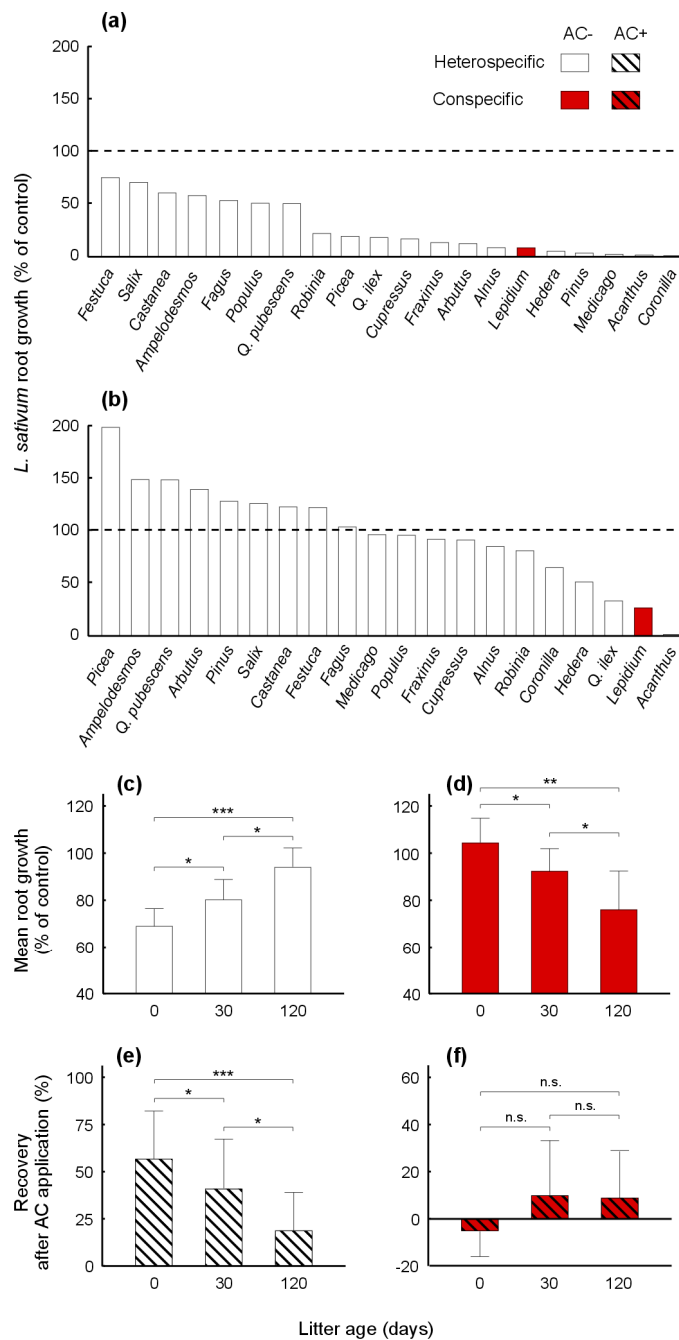


Figure 1. a-b, Effects of 20 litter types on *Lepidium sativum* root growth in percentage compared to control (= 100%) on both undecomposed (a) and 120 days aged (b) materials. c-d, Average root growth of 8 target species either on 19 different heterospecific litters (c) or on their own litter material (d) after 0, 30, and 120 days of decomposition. e-f, Recovery of root growth after Active Carbon (AC) application on heterospecific (e) and conspecific (f) litter at different decomposition ages. Asterisks indicate age-dependent significant differences (***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; n.s., $P > 0.05$; Mann-Whitney-Wilcoxon test)

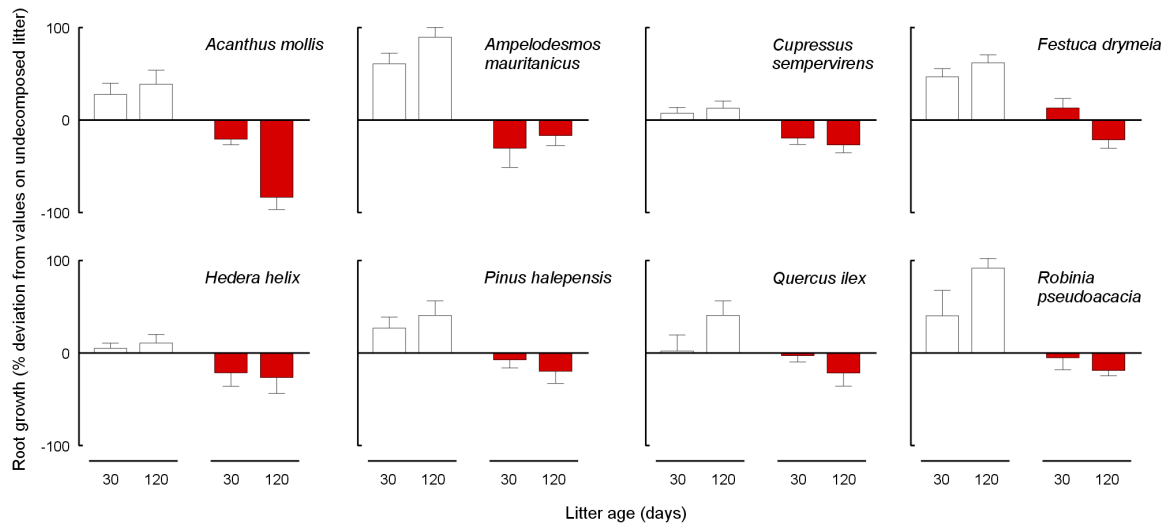


Figure 2. Response of eight target species to litter of heterospecifics (average of seven species) and conspecifics with different ages (0, 30 and 120 days). Values (mean and SD of experimental replicates, $N \geq 5$ observation plates, 5 seedlings each) are expressed as percentage of growth deviations compared to growth on undecomposed litter (= 0%)

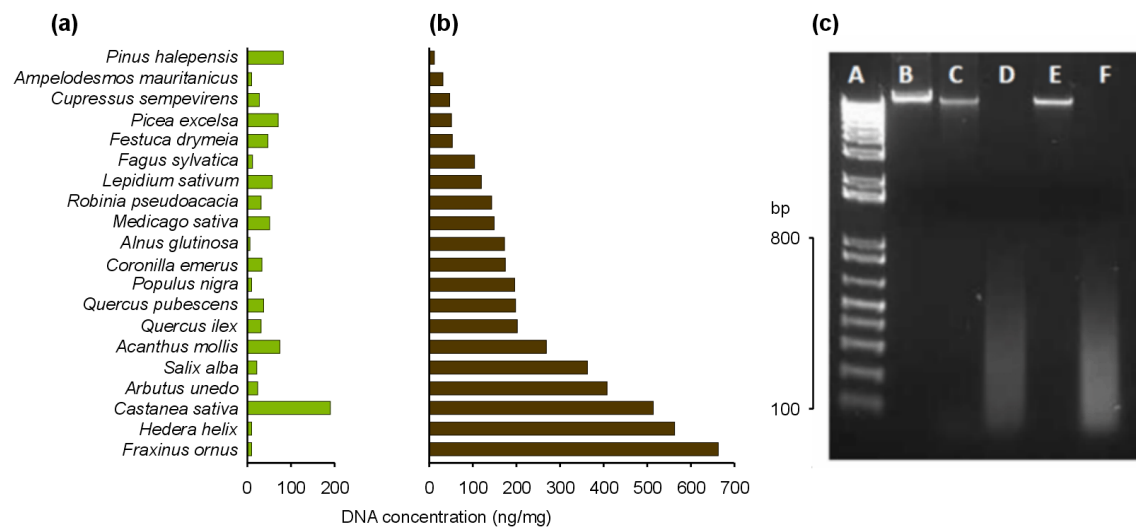


Figure 3. DNA content in decomposing plant litter. **a**, DNA concentration (ng/mg) assessed by UV spectrophotometry in undecomposed materials. **b**, DNA concentration after 120 days of decomposition. **c**, Examples of DNA fragmentation patterns refer to *Acanthus mollis* (C and D, 0 and 120 days, respectively) and *Hedera helix* (E and F, 0 and 120 days, respectively). A and B refer to 1kb ladder plus and lambda DNA, respectively

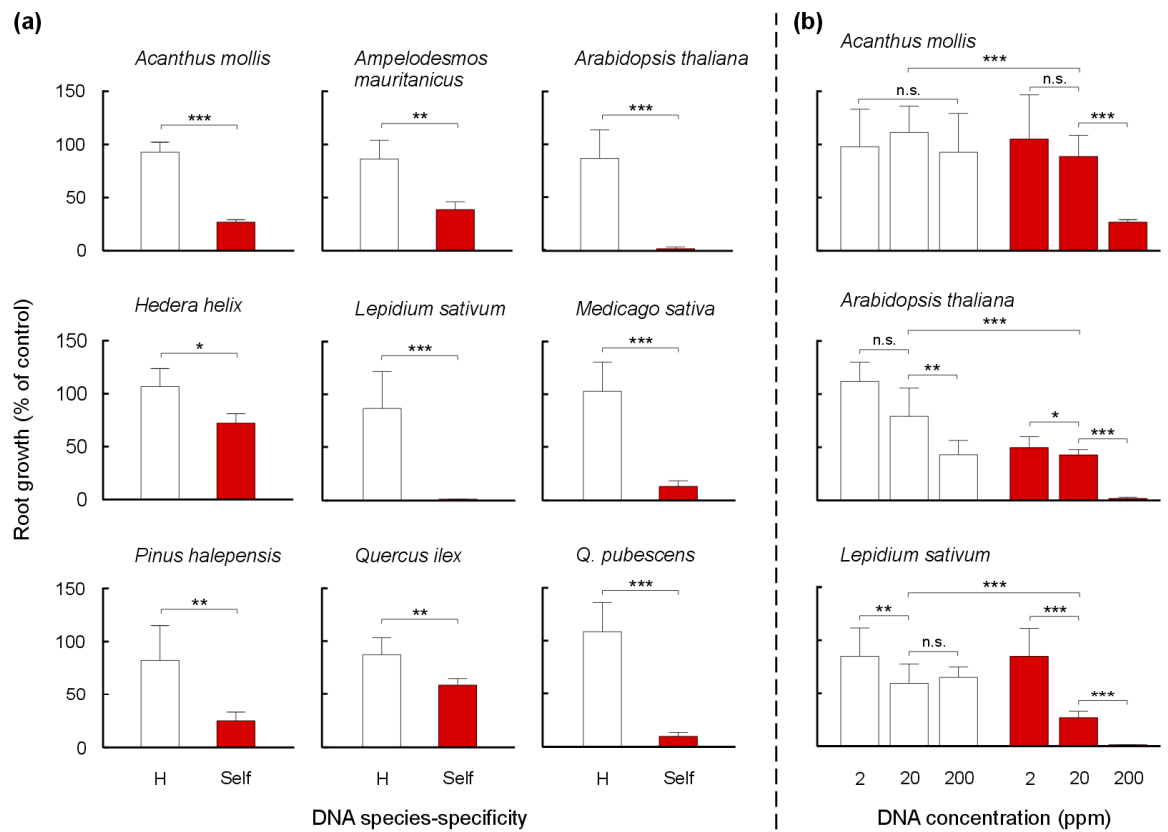


Figure 4. Effects of heterologous and self-DNA on root growth of different plant species. **a**, root growth of nine plant species treated either with a mixture of heterologous (H) or with their own (self) DNA at high concentration (200 ppm), detailed statistics in Supplementary Table S5. **b**, examples of concentration-dependent effects of heterologous (white bars) and self- (red bars) DNA on root growth of three target species. Heterologous DNA sources were *Arabidopsis thaliana* in the case of *Lepidium* target, and *Lepidium sativum* for both treatments on *Arabidopsis* and *Acanthus*. Data are mean and SD, N=20 for each bar; ***, P < 0.001; **, P < 0.01; *, P < 0.05; n.s., P > 0.05; Mann-Whitney-Wilcoxon test



Figure 5. Root observation plates from the litter toxicity experiment. For eight target species representative of different functional groups, plates show root growth of seedlings on either conspecific or heterospecific litter, undecomposed and after 120 days of decomposition, and with or without the addition of activated carbon (AC). Seedlings in control plates are treated with sterile distilled water. Seedlings of *A. mauritanicus* were removed from plates before taking the pictures, to allow displaying root length

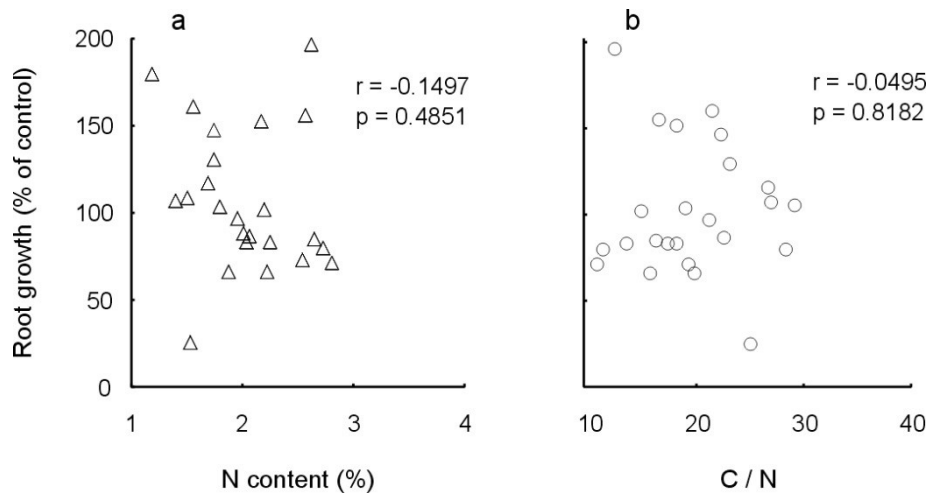


Figure 6. Litter inhibitory effect unrelated to N immobilization. Scatterplot and correlation (Pearson's r and associated P values, $N=24$) between root growth of 8 target species cultured on conspecific litters of three ages (undecomposed, 30 d, 120 d) and litter nitrogen content (a) and carbon-to-nitrogen ratio (b). Non-significant correlation excluded N limitation as a possible causal factor for the observed root growth pattern

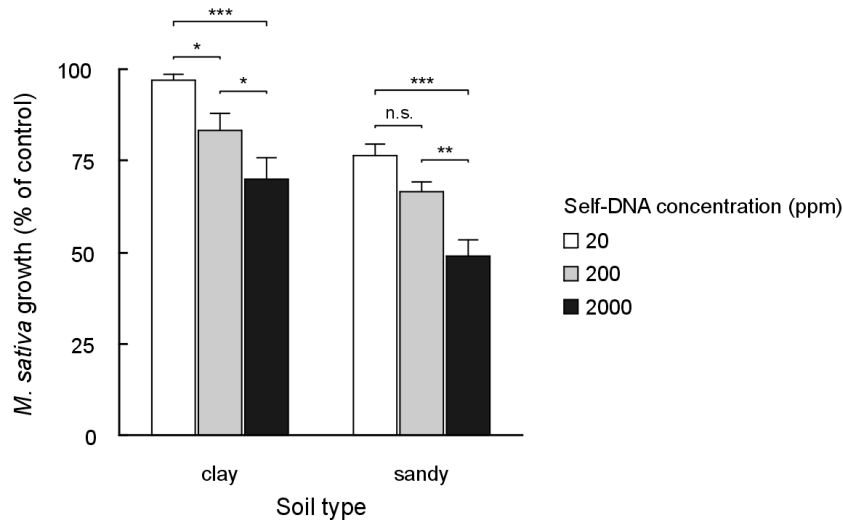


Figure 7. Effect of self-DNA on *Medicago sativa* growth in a greenhouse experiment. *M. sativa* seedlings when exposed to increasing concentration of extracellular self-DNA in two soil types of contrasting texture (sandy and clay). Data refer to mean and s.e.m. of 20 replicated pots (5 seedlings per pot) for each treatment. See Materials and Methods for further information. Differences between soil types and among concentration levels were both statistically significant (two-ways ANOVA, $F_{1,114} = 29.20$, $P < 0.00001$; and $F_{2,114} = 24.01$, $P < 0.00001$, respectively). Pair-wise significant differences are marked with asterisks (***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; n.s., $P > 0.05$; post-hoc Duncan test)

Table 1. Properties of the two soils used in the greenhouse experiment. The experiment was aimed to assess the effect of self-DNA on *Medicago sativa* growth

Soil parameter	Soil type	
	Sand	Clay
Texture (%)		
Sand	45	83.9
Silt	21.5	1.9
Clay	33.5	14.2
pH	8.21	8.41
Organic matter (%)	0.85	0.55
Total N (g/kg)	0.81	0.65
C/N ratio	6.1	5.0
Total CaCO ₃ (g/kg)	189	140
Available P (P ₂ O ₅ , mg/kg)	6.2	48
Exchangeable cations		
K ⁺	0.46	0.53
Mg ²⁺	1.89	0.96
Ca ²⁺	27.6	15.5
Na ⁺	0.35	0.17
Electric Conductivity	0.096	0.188

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Chapter 3: Tests of inhibitory effects of extracellular self-DNA on different organisms

Introduction

Self inhibition or autotoxicity has been reported for several organisms including bacteria (Andersen et al., 1974; Trinick and Parker, 1982), fungi (Bottone et al., 2011), algae (Inderjit and Dakshini, 1994), higher plants (Singh et al., 1999) and animals (Akin, 1966).

The mechanism has been mostly ascribed to the release and accumulation of allelopathic compounds in the growth environment, but the isolation and identification of the molecules which could cause the phenomena have often proved to be difficult. Moreover, none of the proposed compounds and processes has been capable to account for both toxicity and species specificity, failing to provide a general consistent mechanism for the phenomenon of autotoxicity.

The recent observation reported in Chapter 2 of inhibitory effects by extracellular self-DNA in plants provides new perspectives for understanding litter autotoxicity and negative plant-soil feedbacks. While the molecular mechanisms behind these phenomena certainly deserve in-depth investigations, a more basic question arises about the occurrence of similar inhibitory effects of extracellular self-DNA on biological systems other than plants. Therefore, to address this point a set of laboratory experiments was performed on six species selected across different taxonomic groups. These organisms were exposed to increasing concentrations of fragmented self-DNA or heterospecific DNA and their growth performances were compared.

Materials and methods

Experiments included exposure to fragmented self-DNA at different concentrations, fragmented heterologous DNA (*Arabidopsis thaliana*) at the maximum concentration tested for self-DNA, plus the control. Extraction of genomic DNA from each species was performed using standard methodologies. Fragmentation was performed by sonication according to Mazzoleni et al. (2014) in order to obtain fragments mainly distributed in the range between 50 and 1000 bp.

Bacillus subtilis was selected as target Gram-positive bacterium. It was pre-grown on Luria Broth (LB) at 37 °C with agitation (200 rpm). An inoculum was prepared with

10 ml of preculture and 4 ml of LB. Treatments included self-DNA at three concentration levels (40, 200, and 400 $\mu\text{g/ml}$) and heterologous DNA (400 $\mu\text{g/ml}$). All cultures were incubated with agitation (200 rpm) at 37 °C, with three replicates for each treatment and the control. After 24 hrs of incubation, 0.5 ml were taken from each tube and serial dilutions in LB were prepared, from which 100 μl were placed on LB agar plates. Plates were incubated at 37 °C until appearance of colony-forming units (CFU).

Trichoderma harzianum was used as target fungus in a bioassay on spore germination. Fungal spores were produced by pure cultures on potato dextrose agar (PDA). Spores were taken in sterility and diluted to a concentration of $1 \times 10^6 \text{ ml}^{-1}$. Treatments included extracellular self-DNA (8, 80, and 800 $\mu\text{g/ml}$) and heterologous DNA (800 $\mu\text{g/ml}$), with three replicates each. The germination bioassay was performed in ELISA plates (96 wells, 100 μl each), each well coated with 10 μl of liquid 10% PDB substrate, DNA at treatment concentration, fungal spores, and sterile distilled water. Spore germination and germ tube elongation of the conidia were assessed by spectrophotometric analysis and optical microscopy after 20 hours of incubation at 24 °C.

The green microalga *Scenedesmus obliquus* was maintained in Chu's n° 10 medium (Chu, 1942). The cultures were incubated at 25°C under 270 $\mu\text{moles photons m}^{-2} \text{ sec}^{-1}$ light intensity with 16:8 hrs light photoperiod. Treatments of *S. obliquus* were carried out with self-DNA (50 and 500 $\mu\text{g/ml}$) in the culture medium and heterologous DNA (500 $\mu\text{g/ml}$), with two replicates each. Algal growth was assessed by cell counts at the optical microscope after serial dilutions, and growth curves were built for each treatment, until reaching stationary phase (7 days).

Acanthus mollis seedlings were treated with self-DNA (2, 20, and 200 $\mu\text{g/ml}$) and heterologous DNA (200 $\mu\text{g/ml}$), with three replicates. Bioassays were done in vitro by using surface sterile seeds (n=20 in each plate) placed in 9 cm Petri dishes over sterile filter papers imbibed with 4 ml of test solutions. Seedling root length was measured.

Plasmodia of the ameboid protozoan *Physarum polycephalum*, a slime mold widely used in bioassays, were maintained in the dark at 24 °C on 1% agar plates and were fed with oat flakes. Laboratory stocks were subcultured onto new 1% water agar plates and fed by oat flakes. 15 days old massive cultures on Petri plates were used to produce slime mold biomass for total DNA extraction. Tip portions ($17 \pm 5 \text{ mm}^2$) of the plasmodia were taken from stock cultures 8 hours after feeding time and placed on agar substrates at the conditions of maintenance, with three replicated plates for each treatment and the untreated control. Extracted self-DNA (290, 580, and 1060 $\mu\text{g/ml}$) and heterologous

DNA (1060 µg/ml) were applied on 0.2 g of oat flakes placed at the centre of each plate. Pictures of plasmodial growth patterns were taken from each plate every 24 hrs for 96 hrs and used to calculate spreading area size following Takamatsu et al. (2009).

The dipteran *Sarcophaga carnaria* was grown in pure culture on 12 x 12 cm² plates (2 cm height) at 10 °C, fed with ground meat. Treatments included self-DNA (10, 100, and 1000 µg/ml) and heterologous DNA (1000 µg/ml) mixed with 1 g of feeding meat. Three replicated plates, each containing 10 larvae, were prepared for each treatment, plus the untreated control. All plates were incubated in the dark at 10 °C. Development, survival, and time required for the formation of pupae were monitored every 3 days during a 21-days incubation period.

Results

In the bioassays, the effect of all treatments was highly significant. In particular, exposure to either heterologous or self-DNA produced significantly different responses, consistently for all target species (Fig. 1), as showed by the non-significant effect of target species. The application of heterospecific DNA did not produce any significant reduction of species performance (with the exception of *B. subtilis* showing some inhibition also in this case (Fig. 1). Treatments with conspecific DNA resulted in a concentration dependent reduction of species performance. The inhibitory effect at high concentration was consistent for all target species (Fig. 1).

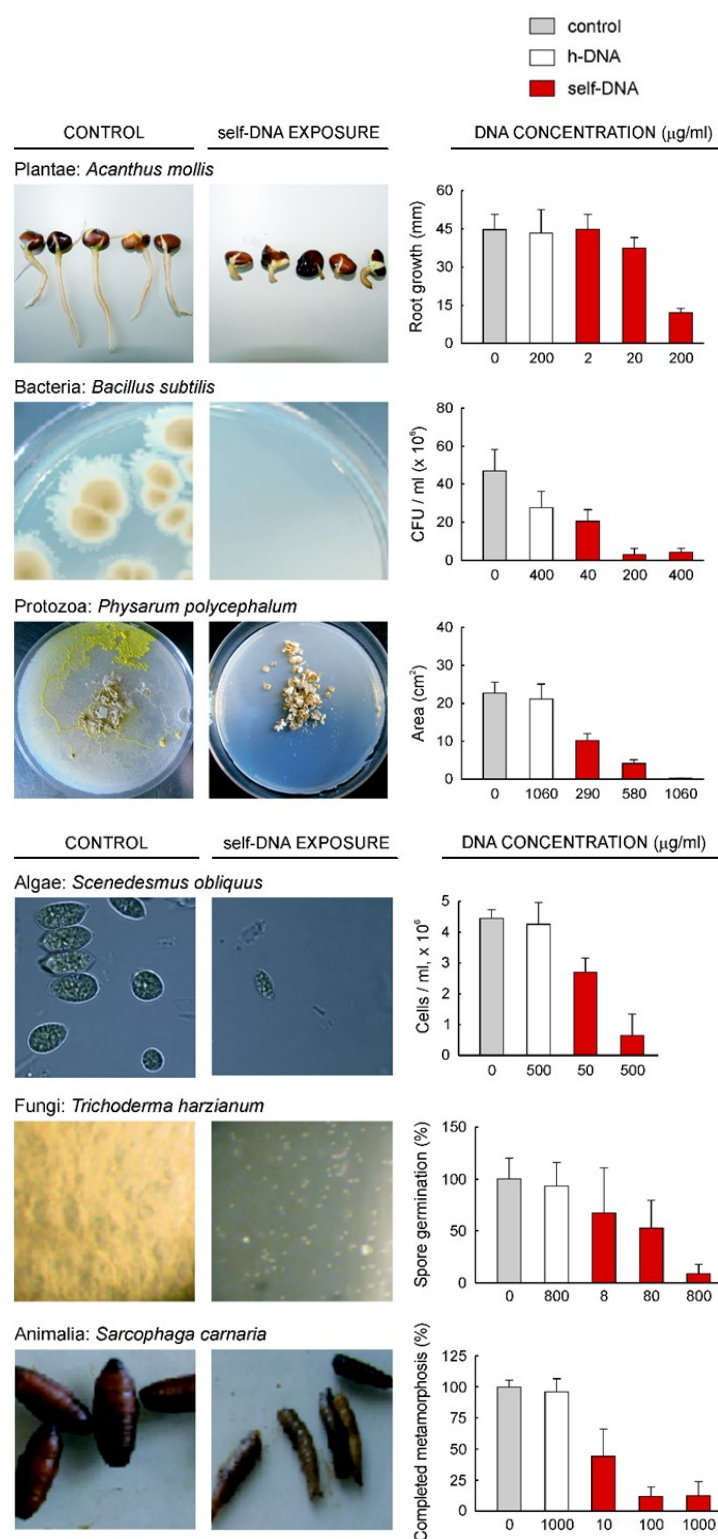


Figure 1. Effects of exposure to heterologous and self-DNA on different organisms. All species show significant concentration dependent inhibitory effects by self-DNA. See Materials and Methods for details on experimental conditions

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Prospettive di studio sulla stanchezza del suolo in agricoltura

Il lavoro sperimentale sulla fitotossicità delle diverse lettieri, confermando studi precedenti (Bonanomi et al., 2006; Bonanomi et al., 2011), ha dimostrato che tutte le lettieri non decomposte provocano forte inibizione della crescita radicale. La fase iniziale della decomposizione della lettiera provoca il rilascio del contenuto cellulare che comprende molecole allelopatiche dall'effetto inibitorio sulla crescita vegetale (Rice, 1984). Queste molecole comunque subiscono una rapida degradazione (Blum, 1998; Blum et al., 2000; Weidenhamer et al., 2013), perdendo velocemente il loro effetto allelopatico (Zucconi, 2003; Bonanomi et al., 2011).

A differenza delle lettieri fresche, gli effetti inibitori delle lettieri decomposte sono risultati specie-specifici. Dopo 120 giorni di decomposizione, il materiale risultante ha perso notevolmente o completamente la tossicità per le piante eterospecifiche. Al contrario, per tutte le specie in esame, la crescita radicale risulta inibita dalla propria lettiera decomposta. Questo risulta coerente con la persistenza per mesi e anni del NF nel suolo osservata sia in ambienti naturali (Hawkes et al., 2013) sia in agroecosistemi (Miller, 1996).

Sia nel suolo sia nella lettiera si accumula DNA (Dilly et al., 2004), capace di resistere alla degradazione per la sua stabilità chimica e l'adsorbimento da parte dei colloidi (Levy-Booth et al., 2007). In questo lavoro è stato quantificato il DNA totale derivato dalla decomposizione vegetale e dalla crescita microbica (Levy-Booth et al., 2007). Questo DNA è frammentato in molecole formate da centinaia a migliaia di nucleotidi.

Il DNA, estratto dalle foglie di pianta e frammentato attraverso sonicazione, ha prodotto evidenti effetti di inibizione specie-specifica. Questi risultati indicano che il proprio DNA risulta tossico per la pianta ed evidenziano il coinvolgimento del DNA extracellulare nel NF.

Studi futuri potranno essere rivolti alla quantificazione del DNA derivato da una singola specie nel suolo al fine di correlare la sua presenza negli agroecosistemi ai fenomeni di stanchezza del terreno.

La stanchezza del terreno indica la condizione di inospitalità del suolo al reimpianto di una singola coltura, non influenzando specie diverse soprattutto se filogeneticamente lontane (Zucconi, 2003). La diminuzione delle produzioni che ne deriva riflette l'incapacità della pianta a nutrirsi nel terreno stanco (Zucconi e Monaco, 1987). Il

problema è prevalente per le specie annuali coltivate in monosuccessione; per alcuni fruttiferi come pesco, melo, pero e vite; oltre che per piantagioni di caffè e tè (Rice, 1984; Chou, 1999; Caboun, 2005; Canals et al., 2005).

La capacità delle cellule radicali di assorbire il DNA (Paungfoo-Lonhienne et al., 2010), insieme alla sua tossicità, può aprire la ricerca a nuovi studi riguardo al coinvolgimento del DNA nella stanchezza del terreno.

Inoltre, la quantificazione nel tempo del DNA nel terreno potrebbe dare indicazioni riguardo alle specie più indicate per le successioni colturali, le consociazioni, i tempi di rotazione e di reimpianto; e, nel caso delle colture in fuori suolo, dare indicazioni sui substrati più adatti, sui loro tempi di sostituzione e su eventuali trattamenti a cui sottoporre gli stessi substrati per un loro riutilizzo economicamente soddisfacente.

Infine, restano da chiarire i meccanismi funzionali dell'effetto inibitorio del DNA extracellulare anche per comprendere i motivi della differente sensibilità di diverse specie e l'interessante problematica delle specie "aliene" invasive in ecosistemi diversi da quelli di origine.

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